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BIOLOGY OF THE TREPONEMATOSES

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OF THE

TREPONEMATOSES

Based on Studies Carried Out at the International Treponematosis Laboratory Center of the Johns Hopkins University under the Auspices of the World Health Organization

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Preface

This monograph is based on the results of investigations carried out at the International Treponematosis Laboratory Center, which was established in 1950 in the Department of Microbiology of the Johns Hopkins University under the joint auspices of the World Health Organization and the Johns Hopkins School of Hygiene and Public Health. WHO has stimulated the work of the Center from the very beginning, lending its support and encouragement to the studies undertaken both in the field and in the laboratory. Thus, the Center's activities have formed part of WHO's programme for the co-ordination of research in treponematosis control.

Support in the form of grants has also been received from the National Institutes of Health, United States Public Health Service, and the Whitehall Foundation Inc. of New York. Over the years, the International Health Division of the Rockefeller Foundation has given considerable financial aid to the work of the Center, and a number of other organizations and individuals have contributed lesser but nevertheless significant degrees of support; among these may be mentioned particularly the late Mr George Moffet, and the Lederle Laboratory Division, American Cyanamid Company.

Finally, the generous attitude of the Johns Hopkins University in providing facilities at a cost difficult to estimate but certainly greater than all the outside contributions combined has played no small part in making possible this series of studies.

CONTENTS

APPENDICES

Taken the world over the treponemal diseases—syphilis (venereal and endemic), yaws, and pinta—are among the major afflictions of mankind In few countries is treponematosis a minor health problem; in many countries it is a major one.

Penicillin has rendered the control of the treponemal diseases a practical possibility for the first time, their complete elimination is now no longer a fantastic objective But years of intelligent and patient application of present knowledge will be required to bring about in most countries even partial control

Always, too, inherent in an interacting biological system such as that between the treponemes and man, their natural host, are opportunities for either temporary or permanent evolutionary changes to occur which could upset whatever favorable advantage man enjoys.

In the formulation of long-range plans directed to the control of the treponematoses it behoves us therefore first to make use of all the knowledge of the fundamental biology of the disease now available and secondly to press the search for new knowledge which man might need to retain his advantage over the treponeme.

For a number of years a group of investigators in association with the senior author has been studying one or another aspect of the treponemal diseases, more recently this group has served as the International Treponematosis Laboratory Center under the auspices of the World Health Organization. It is mainly the studies and observations of these investigators that are recorded here, although in attempting to present the newer knowledge of the fundamental biology of the treponematoses no work from other sources which would help complete the picture has been intentionally omitted The monograph is arranged in three parts Part I comprises six chapters dealing with various aspects of the fundamental biology of the treponematoses in general, Part II comprises three chapters in which a comparative study of strains of treponemes newly isolated from various parts of the world is presented, and Part III is a single chapter summarizing the principal observations presented in Part I and Part II.

In many respects our view of the treponemal diseases has changed considerably over the past few years, and it is the observations and principles underlying these changing concepts with which this monograph is

mostly concerned. In a sense, therefore, it does not supersede but rather builds upon the splendid summaries of knowledge in this field, among which are those by Chesney,[2] Noguchi,[15] Mulzer;[14] Sobernheim, Bruck, Prigge, and Laubenheimer in Kolle, Kraus & Uhlenhuth's handbook of pathogenic organisms;[10] Matsumoto;[11, 12] Bulloch,[1] and Dawson[3] in the British Medical Research Council's *System of bacteriology*; Gastinel & Pulvenis,[4] Kolle & Hetsch,[9] various contributions to the symposium of the American Association for the Advancement of Science;[13] Hudson's monograph on treponematosis,[8] and the reviews of pinta by Holcomb,[7] of endemic syphilis by Grin[5] and of the treponematoses as a whole by Guthe & Willcox[6]

While of necessity much knowledge of the treponematoses has been gained from a study of laboratory animals, in the background is always the important question of the extent to which such observations can be translated into terms of the disease in human beings. Recognizing that caution and good judgement must be exercised in translations of this sort, it will nevertheless be regarded as an obligation to suggest interpretations of laboratory-acquired data in the light of the clinical and epidemiological problems of the treponemal diseases.

The authors have given much thought to the selection of a suitable title for this monograph While "Biology of the Treponematoses" is open to certain objections from a strictly semantic point of view, it is felt that it conveys better than any other alternative title considered the breadth of the studies reported therein These are concerned not only with the infecting organism but also with many fundamental aspects of the disease processes included under the term "treponematoses", as well as their pathogenesis, their epidemiology, their immunology and their microbiology.

* * *

Throughout the period during which the senior author has been the principal investigator, he has been fortunate in having the collaboration of a succession of splendid younger investigators. References to their work appear throughout the text, but countless hours of detailed study and much background material have been contributed in such a way that it is impossible to give adequate credit individually It is with sincere appreciation as well as with a warm sense of personal debt that acknowledgements are made to the following former or present associates: Drs George M. Saunders, Henry W. Kumm, A. A. Peat, H. M. Johnston, J. I. Rerrie and L E. Arnold (all of the one-time Jamaica Yaws Commission); Drs William L. Fleming, Elaine Updyke, Fred C. Kluth, Charlotte McLeod, Mary Cumberland Yurchenco and Miss Nancy Brayton Krieble (during the period prior to the outbreak of the Second World War); Drs David H. Hollander, Huan-Ying Li, Robert A. Nelson, jr., Katherine Schaeffer, Paul Hardy,

Morton Weber, Justina H Hill, A. S. Khan and Miss Ellen Nell (during the period from the end of the Second World War to the present time). In addition, along the way, inestimably valuable advice has been received from Drs Alan M Chesney, Joseph Earle Moore, Manfred M. Mayer and Abraham G. Osler

Credit is due to the World Health Organization and in particular to Dr Thorstein Guthe, Chief of its Venereal Diseases and Treponematoses Section, and his former associate, Dr Frank W. Reynolds, for suggesting the idea of this monograph and for stimulating its preparation.

To Dr David H. Hollander, the co-author, goes the appreciation and respect due to a partner who has shared the burdens as well as the satisfactions that have accompanied the preparation of this volume. Mrs Anne Thompson and Miss Virginia Lee Robinson were responsible for the painstaking preparation of the typescript

Finally, the authors are indebted to the Columbia University Press and to the editors and publishers of the following journals for permission to reproduce certain tabular material *American Journal of Hygiene* ; *American Journal of Syphilis, Gonorrhea, and Venereal Diseases* , *Applied Microbiology* ; and the *Bulletin of the Johns Hopkins Hospital*

THOMAS B. TURNER

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Part I

BIOLOGY OF TREPONEMAL INFECTIONS

Chapter 1

SOURCES OF STRAINS STUDIED

Clinical and Epidemiological Entities

Syphilis and yaws as distinct disease syndromes have been recognized for over 400 years. Speculation concerning their relationship considerably antedates the discovery of the etiologic agents, *Treponema pallidum* and *T. pertenue*, in 1905, the former by Schaudinn & Hoffman [16, 18] and the latter by Castellani [4] While this speculation still continues, few informed persons deny that clinical and epidemiological differences do, in fact, exist between the naturally occurring diseases, and most will agree that distinguishing names for the two syndromes are both useful and biologically justifiable.

Since these two diseases obviously have many features in common, the real question is What is the basis for the observed differences, and how stable are the distinguishing characteristics ? Ultimately the question of whether two disease syndromes are the same or different becomes a philosophical one and perhaps even a semantic argument, it is probable that armchair dialectics have contributed about all that may be expected to the syphilis-yaws problem, and there remains the need for fundamental comparative data under more or less controlled conditions

Another disease syndrome that comes clearly within the general category of the treponematoses is pinta, or mal del pinto Since the clinical and epidemiological pattern of this disease does not closely resemble syphilis or yaws, its biologic relationship to these syndromes was not suspected until about twenty-five years ago when an unusually high incidence of positive Wassermann reactions in pinta patients was commented upon by Menk,[12] and by González-Herrejón & Pallarés [7] In 1938 Sáenz, Grau Triana & Armenteros [15] found treponemes in a patient with pinta, and León-Blanco [10] and others subsequently showed that treponemes resembling *T. pallidum* were regularly present in certain types of pinta lesions

The three foregoing syndromes represent perhaps the most clear-cut entities within the treponematosis group Yet there are many others that also clearly belong within the group, but which for lack of extensive study or because of marginal differential criteria have not achieved standing as

distinct clinical entities. Among this group may be mentioned bejel, which occurs characteristically among nomadic tribes of the hot dry areas of Asia Minor and the Mediterranean area; endemic syphilis, in sections of the Balkans; dichuchwa, in Bechuanaland and adjacent areas; njovera, in Southern Rhodesia; and siti, in British West Africa All these are basically endemic syphilitic infections acquired in infancy.

In addition to these syndromes observed in man, there is a naturally occurring disease of domestic rabbits, known in the medical literature as venereal spirochetosis of rabbits,[1, 14] which is biologically related to the treponematoses of man.

In this monograph comparative data on the behavior of treponemes of most of these syndromes will be presented, and an attempt made to determine wherein they resemble one another or differ.

The Experimental Approach

It is only possible to carry out adequately controlled experiments on disease in man under exceptional and almost always difficult circumstances And so it has been with the treponematoses. Many observations of great value have been made on the treponemal diseases of man; but in general these have been of a descriptive nature, or else—as in the therapeutic use of penicillin—so striking that there is no question of the validity of the results.

There remain, however, many questions of the fundamental biology of the disease which can scarcely be studied in man under controlled conditions, and it is necessary, therefore, to turn to the laboratory for clues as to the answer to these questions The limitations of such an approach are readily conceded, yet it is the only one which at the moment gives promise of worth-while results

In the laboratory, it is practicable to study the behavior of different strains and species of treponemes in the same host species maintained under virtually identical conditions; or by working with the same strain of treponeme to observe its behavior in a host species subjected to various modifying procedures. Even here, however, the relatively leisurely pace of the evolution of the treponemal disease process poses difficulties, and at the least makes it necessary to carry out experimentation on a time-scale much too extended to be fully consonant with the time-scale for the development, maturation and senescence of the individual human investigator.

Then, too, while one is not handicapped in experimental treponemal research by some of the enormous difficulties under which the investigator of cancer or leprosy, for example, must work, inability to cultivate *in vitro* the etiological agents of the treponemal diseases imposes serious limitations, particularly in attempts to study the metabolism of the treponeme or its antigenic structure. Such limitations, however, properly serve as a challenge to the investigator and indeed at times become ends in themselves.

Emphasis will be placed on what has been accomplished, without undue preoccupation with investigations in which failure has been the result

Definitions

In every laboratory a scientific jargon—a kind of shorthand or abbreviated language—develops which is useful in conveying ideas with the least number of words. The reader will be spared most of these, but a few expressions, of necessity, recur so frequently that it seems permissible to define them here and continue to use them throughout this monograph. The principal examples are the following·

Syphilis treponeme (or yaws treponeme, bejel treponeme etc.)—One or another strain of *T. pallidum*, *T. pertenue* or *T carateum* originally derived from a typical case of syphilis, yaws, bejel or endemic syphilis or pinta Since much of the experimental work in this laboratory was carried out with the Nichols strain of *T. pallidum*, this strain will usually be the one used when no other identification is given The word treponeme as used here denotes a pathogenic spirochete belonging to the genus *Treponema*.

Cuniculi infection, cuniculi treponeme—The disease " spontaneous venereal spirochetosis of rabbits " originally described by Ross [14] and Bayon,[1] and the treponeme, *T cuniculi*, which is the etiological agent of the disease.

Wassermann antibody—The substance in the serum of human beings or experimental animals infected with treponemes that is measured by the standard serological tests using lipoidal antigens The term " reagin " is synonymous with Wassermann antibody

Standard serological tests (STS)—Those tests which detect Wassermann antibody by flocculation or complement-fixation reactions with lipoidal antigens, including Wassermann, Kahn, Eagle, Mazzini, Kline, Hinton, and other similar tests These tests are grouped together in contradistinction to tests which measure other antibody, for example immobilizing or agglutinating antibody (For further discussion of this subject see Chapter 5.)

Cardiolipin antigen and the VDRL test are included, for the purposes of this monograph, in the category of standard serological tests

TPI test—Treponemal immobilization test

TPA test—Treponemal agglutination test

Darkfield examination darkfield positive, darkfield negative—The microscopic examination of material using darkground illumination. The material is said to be darkfield positive, or darkfield negative, depending on whether or not treponemes are demonstrated by this method.

Infectivity test—Unless otherwise specified, this term refers to the inoculation of material into an animal in order to determine whether the inoculated material contains virulent treponemes Such tests are commonly made by inoculating the material into one or both testes of two normal

rabbits, which are then observed for 90 days The development of characteristic lesions, in which treponemes can be demonstrated by darkfield examination, constitutes a positive test. While a positive infectivity test is proof that the inoculated material contained rabbit-virulent treponemes, a negative test does not necessarily exclude their presence, although it constitutes valuable evidence on that point particularly with a well-adapted laboratory strain of syphilis treponemes.

Normal or negative animal—These terms are colloquial but useful. Strictly speaking there is probably no such thing as a "normal" animal. As used here it means simply that the animal has not previously been infected with the particular agent under discussion.

The term "negative" is used to indicate that the animal shows no signs or symptoms suggestive of the specific disease process in question.

Classification of spirochetal organisms

It is of the essence of the thoughtful biologist that he is at once busily engaged in cataloguing the individuality of living forms, while at the same time he is seeking for common denominators that will reveal relationships and similarities hitherto hidden

In the microscopic world of the bacteria three principal morphologic categories have long been distinguished. These three, or modifications thereof, are the spherical forms, the rod-like or perhaps more strictly speaking the cylindrical forms, and the helical, or those that have a spiral shape. Because perhaps the spiral micro-organisms have not been as well studied as many representatives of the other two groups, they are less familiar and a little more mysterious to most medical biologists. There is a tendency, therefore, stemming perhaps from this ignorance, to regard all these spiral micro-organisms as being in some way related biologically. And indeed they are, in that they have a common form , but it is unwise to go much further than this, except where modern methods provide a solid basis for conjecture Unfortunately, for the course of easy assumptions, studies have frequently revealed differences rather than similarities when varieties of spirochetal organisms have been compared

In the last edition of Bergey's *Manual of determinative bacteriology*,[3] which represents the consensus of informed American opinion, essentially the following classification of the spiral organisms (not including the vibrios which are only slightly spiral) is given·

ORDER V *Spirochaetales* Buchanan

FAMILY I *Spirochaetaceae* Swellengrebel
Spirals 30-500 μ, with definite protoplasmic structures
Genus I *Spirochaeta* Ehrenberg—No periplast or cross-striations
Genus II *Saprospira* Gross—Free living, periplast and cross-striations
Genus III *Cristispira* Gross—Parasitic, periplast and cross-striations

FAMILY II *Treponemataceae* Schaudinn

Spirals 4-16 μ, without obvious protoplasmic structure

Genus I *Borrelia* Swellengrebel—Stain easily

Genus II *Treponema* Schaudinn—Anaerobes, stain with difficulty

Genus III *Leptospira* Noguchi—Aerobes, stain with difficulty

There is no conclusive evidence indicating a close biological relationship between the *Treponema*, and either the free-living *Spirochaeta*, *Saprospira* and *Cristispira* on the one hand, or the more dependent genera such as the *Borrelia* and *Leptospira* on the other Indeed, there is much bacteriological, immunological and pathological evidence which suggests that the *Treponema* are no more closely related to these other genera of spiral organisms than to many micro-organisms that are spherical or rod-shaped.

It would be attractive to speculate about such relationships in the light of modern genetics, and to conceive the spiral form as a thread linking all these groups, but there is no evidence to support such a linkage and one can scarcely afford the luxury of this idle speculation if one is not prepared to apply some well-established technical methods to the study of the problem.

So let us, therefore, dismiss from consideration for the time being other groups of spiral organisms not morphologically identical with the *Treponema*, on the basis that there is little or no evidence indicating a useful degree of biological relationship between these other groups and the organisms with which this monograph is primarily concerned

Classification within the Treponema *group* No entirely satisfactory classification of organisms belonging to the *Treponema* group is now available Unfortunately, despite much application to this problem, we have no new or improved scheme to propose, although the subject will be discussed again in Chapter 10 As a working classification we have adopted the following, which clearly rests on clinical and epidemiological considerations

1. *Human pathogens primarily*· Includes the causative agents of
   (*a*) syphilis (*T pallidum*)
   (*b*) yaws (*T pertenue*)
   (*c*) pinta (*T carateum*, sometimes called *T herrejoni*, *T. pictor*, *T americana*, *T. discromoderma*, or *T. pintae*)
   (*d*) bejel and other non-venereal syndromes which clinically and epidemiologically appear to be closely related (*T pallidum*, or bejel treponemes etc)

2 *Animal pathogens primarily*· Includes the causative agent of non-venereal spirochetosis of rabbits (*T. cuniculi*).

3 *Human saprophytes primarily*: Includes the mouth spirochetes (*T macrodentium* and *T. microdentium*) and related spirochetes often present about the anus and in fecal material. Included here are also

the culture spirochetes of which the best known are the Reiter; Kroo; Noguchi, Kazan; and Nichols strains.

Strains of treponemes studied

Over the years some 76 different strains of *Treponema* have been isolated, and most of them propagated for a time at least in animals, at the International Treponematosis Laboratory Center—the senior author's laboratory. We shall use the term " strain " here in its commonly accepted sense as being a collection of individual *Treponema* which were obtained originally from the same source, commonly a human being or an animal with a naturally occurring infection, and propagated in straight-line succession.

Some of these strains were isolated primarily for the purpose of making comparative studies of one sort or another, others were isolated during the study of some other aspect of the biology of the treponematoses In most instances, however, care was taken to assure that a particular strain was obtained from a source which could be regarded as typical of the clinical disease to which medical custom has attached a distinguishing name Thus, with rare exceptions the strains of syphilis spirochetes and yaws spirochetes were secured from patients selected precisely because they presented classical evidence of the respective disease syndromes according to the clinical and epidemiological criteria of informed observers. The same statement can be made for the other treponemal syndromes studied, although often in these instances clinical and epidemiological criteria may have been less clear-cut.

In describing below the strains studied *in* this laboratory, therefore, no apologies will be made for designating these as syphilis or yaws strains (*T. pallidum* and *T. pertenue*, respectively) as the case may be. We have, however, in the recent studies in collaboration with the World Health Organization, designated these strains by the locality of origin in an effort to divest ourselves of any prejudicial notion, in so far as this is possible, concerning the biological inter-relationships existing among these strains.

Of the 76 strains studied, 39 were from patients with a clinical diagnosis of syphilis; 20 were from patients with a clinical diagnosis of yaws; 3 from patients with bejel; 8 from endemic syphilis or one of the treponemal syndromes which is recognized by a local name only; and 6 strains were isolated from a naturally occurring disease in rabbits: venereal spirochetosis of rabbits. In addition, treponemes from patients with pinta were recovered three times in the initial passages in hamsters, but the strains were lost on subsequent passage.

These 76 strains were isolated at four different periods. The first group in point of time comprised 21 strains from syphilis and yaws patients isolated during the years 1932-35 by the senior author and his associates—at that time on the Jamaica Yaws Commission. All strains in this group were

TABLE 1A STRAINS OF TREPONEMAL ORGANISMS ISOLATED AND PROPAGATED IN LABORATORY ANIMALS STRAINS ISOLATED FROM PATIENTS LIVING IN JAMAICA, B W I

Strain designation	Date of transfer to laboratory animals	Patients' initials	Clinical diagnosis	Incubation period in 1st animal passage (days) [a]	Total animal passages observed	Still available in this laboratory [b]
YA	30 3 32	S C	Yaws—generalized	28	47+	Yes
Undesignated [c]	2 5 32	M S	,,	35	3	No
YB	30 11 32	J D	,,	—	10	,,
Undesignated	6 3 33	L P	,,	50	1	,,
,,	23 3 34	A W	,,	54	1	,,
,,	27 4 34	E S	,,	67	1	,,
,,	2 5 34	M C	,,	52	3	,,
YC	21 6 34	A W	,,	40	9	,,
YD [d]	6 7 34	C T		43	25+	Yes
YE	27 7 34	M S	,,	60	6	No
YF	24 8 34	R W	,,	—	5	,,
YH	7 2 35	L W	,, [e]	19	15	,,
YK	12 2 35	A G	,, [e]	26	6	,,
S1	19 8 32	H S	Primary syphilis	53	10	No
S2	10 2 33	J R		60	4	,,
S3	24 11 33	A R	Secondary syphilis [f]	30	8	,,
S4	1 12 33	K P	Primary syphilis	35	8	,,
S5	16 2 34	L D	,,	30	8	,,
S6	18 1 34	L H	Secondary syphilis [f]	40	22	,,
S8	2 2 34	S M	,, [f]	48	8	,,
S10	10 7 34	A F	,, [f]	23	8	,,

[a] All isolations were made by intratesticular inoculation of rabbits, except where noted
[b] As of 1 September 1955
[c] "Undesignated" refers to strains not propagated in serial passage
[d] See Chapter 7 for history of strain YD and designations YD-pre-1949 and YD-post-1949
[e] Isolations made on granulating wound of rabbit by *Hippelates* fly] transmission (Kumm & Turner ')
[f] Transfer material from lymph node

isolated from patients who were permanent residents of Jamaica, B W.I.; 8 strains were from adults with characteristic histories, including strong presumption that the disease was acquired by sexual exposure, and physical signs of early syphilis, and 13 strains were from persons, mostly children, in whom the epidemiological and physical findings were characteristic of yaws

the culture spirochetes of which the best known are the Reiter; Kroo; Noguchi; Kazan, and Nichols strains.

Strains of treponemes studied

Over the years some 76 different strains of *Treponema* have been isolated, and most of them propagated for a time at least in animals, at the International Treponematosis Laboratory Center—the senior author's laboratory. We shall use the term " strain " here in its commonly accepted sense as being a collection of individual *Treponema* which were obtained originally from the same source, commonly a human being or an animal with a naturally occurring infection, and propagated in straight-line succession.

Some of these strains were isolated primarily for the purpose of making comparative studies of one sort or another, others were isolated during the study of some other aspect of the biology of the treponematoses In most instances, however, care was taken to assure that a particular strain was obtained from a source which could be regarded as typical of the clinical disease to which medical custom has attached a distinguishing name Thus, with rare exceptions the strains of syphilis spirochetes and yaws spirochetes were secured from patients selected precisely because they presented classical evidence of the respective disease syndromes according to the clinical and epidemiological criteria of informed observers. The same statement can be made for the other treponemal syndromes studied, although often in these instances clinical and epidemiological criteria may have been less clear-cut

In describing below the strains studied in this laboratory, therefore, no apologies will be made for designating these as syphilis or yaws strains (*T pallidum* and *T. pertenue*, respectively) as the case may be. We have, however, in the recent studies in collaboration with the World Health Organization, designated these strains by the locality of origin in an effort to divest ourselves of any prejudicial notion, in so far as this is possible, concerning the biological inter-relationships existing among these strains.

Of the 76 strains studied, 39 were from patients with a clinical diagnosis of syphilis; 20 were from patients with a clinical diagnosis of yaws; 3 from patients with bejel; 8 from endemic syphilis or one of the treponemal syndromes which is recognized by a local name only; and 6 strains were isolated from a naturally occurring disease in rabbits, venereal spirochetosis of rabbits. In addition, treponemes from patients with pinta were recovered three times in the initial passages in hamsters, but the strains were lost on subsequent passage

These 76 strains were isolated at four different periods. The first group in point of time comprised 21 strains from syphilis and yaws patients isolated during the years 1932-35 by the senior author and his associates—at that time on the Jamaica Yaws Commission. All strains in this group were

A second group of isolations was made during the years 1937-41 from two sources 18 strains from patients with typical signs of early syphilis, all of whom lived in Maryland; and 6 strains from as many domestic rabbits which were observed by chance in this laboratory and which had characteristic evidence of cuniculi infection All isolations were made in this laboratory by the senior author and his associates. Pertinent data on this group of strains are given in Table Ib Strains from human beings were designated by the initials of the patient from whom they were obtained, together with the year of isolation, as for example K. C. 37; strains from rabbits with cuniculi infections were designated cuniculi A, cuniculi B, and so on

A third group of 9 strains was isolated during 1947 and 1948. All were from patients with syphilis, 6 patients lived in Maryland, 2 in the Los Angeles area of California, and one in St Louis, Missouri As in the previous groups, all isolations were made by intratesticular inoculations of rabbits Strains were designated in a manner similar to that adopted in the case of the preceding group Pertinent data on these strains are shown in Table Ic. It will be noted that several of these strains were obtained from material secured by biopsy, rather than from cutaneous lesions

A fourth group of 22 strains was isolated during 1950-55 by the authors and their associates from patients living in various parts of the world

TABLE Ic STRAINS OF TREPONEMAL ORGANISMS ISOLATED AND PROPAGATED IN LABORATORY ANIMALS STRAINS OF SYPHILIS ISOLATED IN THE UNITED STATES OF AMERICA

Strain designation	Date of transfer to laboratory animals	Clinical diagnosis	Incubation period in 1st animal-passape (days)	Total animal passages observed	Available in this laboratory
A H 47	12 11 47	Secondary syphilis	31	3	No
F R 47	17 11 47	Secondary syphilis	49	3	"
St Louis 47	27 11 47	Early syphilis (node) [a]	14	17	"
L J W 47	5 12 47	Secondary syphilis	25	2	"
H W 48	21 1 48	Secondary syphilis (liver biopsy) [b]	55	2	"
W M 48	2 11 48	Late syphilis (liver biopsy) [b]	65	4	.
C C 49	17 1 49	Late syphilis (node) [c]	39	2	.
Cal I	19 10 48	Primary syphilis [d]	—	2	"
Cal II	21 12 48	Primary syphilis [d]	—	6	"

[illegible]

For identification, these strains, pertinent details concerning which are given in Table IA, were designated *S1*, *S2*, etc., if they were isolated from a yaws patient. All transfers from patients to rabbits were made by inoculation of material into the body of the rabbit's testis, except in the case of two strains, YH and YK, which were transmitted from man to rabbit through *Hippelates* flies. The isolation of these two strains has been described in more detail by Kumm & Turner [9]

TABLE IB STRAINS OF TREPONEMAL ORGANISMS ISOLATED AND PROPAGATED IN LABORATORY ANIMALS STRAINS ISOLATED FROM PERSONS LIVING IN THE USA, AND FROM NATURAL INFECTIONS OF RABBITS *

Strain designation	Date of transfer to laboratory animals	Clinical diagnosis	Incubation period in 1st animal passage (days)	Total animal passages observed	Available in this laboratory
K C 37	1 3 37	*Secondary syphilis*	43	3	No
S M 37	14 9 37	Primary syphilis	36	5	"
D T 37	25 10 37	Secondary syphilis	82	3	"
S R 37	2 11 37	Primary syphilis	50	3	"
F S 37	16 11 37	Secondary syphilis	34	3	"
P A 37	3 12 37		–	3	"
N P 37	14 12 37	(blood)	37	4	"
B T 38	3 1 38	Primary syphilis	–	3	
E C 38	10 1 38	Secondary syphilis	40	2	"
V M 38	18 1 38	Primary syphilis	63	3	
L W 38	24 1 38		36	3	"
C J 39	12 9 39	Secondary syphilis	30	6	"
A G 39	18 9 39		39	3	"
M S I 39	13 10 39		40	18	Yes
M S II 39	18 10 39		60	4	No
L W 39	1 11 39		47	5	
M S S 39	30 11 39		32	4	"
M J 41	21 11 41	(blood)	42	4	"
Cuniculi A	28 12 39	Rabbit non venereal spirochetosis	26	37	Yes
Cuniculi B	3 2 40		24	14	No
Cuniculi C	28 10 40	"	30	2	"
Cuniculi D	22 1 41		20	1	"
Cuniculi E	22 1 41	"	20	1	"
Cuniculi F	24 10 41	"	32	1	"

* All strains were isolated from patients living in Maryland unless otherwise noted, all isolations were made by intratesticular inoculation of rabbits

Isolations were made under the auspices of the World Health Organization with the collaboration of scientists who made the initial transfers from patients to animals in the respective areas Additional details of these isolations are given in Appendix 1 (page 269)

Altogether during this last period 4 strains were isolated from patients with typical syphilis, 7 strains from typical yaws; 3 strains from patients with bejel, and 8 strains from individuals with one or another of the treponematoses which locally are known by other names Pertinent data on these strains are shown in Table Iᴅ, but reference will be made to them again in Chapters 7, 8 and 9.

Throughout this period, the well-known Nichols strain of *T. pallidum* was used in the nature of a " standard " laboratory strain, and many of the special studies were made with this strain. The Nichols strain was isolated in 1912 by Major H J Nichols of the United States Army from the spinal fluid of a patient with recurrent neurosyphilis,[13] and has been maintained more or less continuously in laboratory animals since that time. About every decade, one or more accidental laboratory infections of human beings have been observed with this strain, thus attesting to its pathogenicity for man despite long propagation in laboratory animals (See Chapter 7)

Methods of Isolation of Treponemal Strains

Types of lesions used

In most instances skin lesions, showing numerous treponemes, of patients with characteristic signs of the disease under study were selected as sources of transfer material In yaws patients this was ordinarily a frambesiform lesion, and in syphilis patients a penile chancre or a skin papule of the secondary stage.

The lesion was grasped about the base with a hemostat to fix the lesion and to effect a degree of hemostasis The surface of the lesion was washed with sterile water or saline and was gently abraided Usually the serum, which oozed freely, was rich in actively motile treponemes Serum from the lesion was collected with a capillary pipette or small syringe with or without slight dilution in normal saline solution The addition of 10% inactivated normal serum to the saline is desirable since treponemes appear to survive poorly in saline As soon as 0 2-1 0 ml of material was collected it was inoculated into one or more laboratory animals.

Inoculation of rabbits

All isolations prior to 1950 were made by direct transfer of infective material to rabbits, usually by intratesticular inoculation. It had previously

TABLE 10 STRAINS OF TREPONEMAL ORGANISMS ISOLATED AND *PROPAGATED* IN LABORATORY ANIMALS
STRAINS ISOLATED *FROM* PERSONS LIVING IN *VARIOUS* PARTS OF THE WORLD

Strain *designation*	*Date* of transfer to laboratory animals	Source of strain: Patients' *initials* and age	Source of strain: Transfers (See reference *numbers in* Appendix 1)	Source of strain: Clinical diagnosis	Incubation period in 1st animal passage (days): *Rabbit*	Incubation period in 1st animal passage (days): Hamster	*Total* animal passages observed	Available *in this* laboratory [a]
Syria A	6 5 50	A M 6 years	1	Bejel	33 *testis*, 27 back	43, 30, 32	31	No
Syria B	6 5 50	W D (child)	2	Bejel	67	—	35	Yes
Bosnia A	5 9 50	K A S 35 years	3	Endemic syphilis	60	—	33	″
Bosnia B	5 9 50	N G G 38 years	4	″	77	—	33	″
Baghdad A	30 12 50	S H 20 years	5	*Venereal* syphilis	35 back	—	47	″
Baghdad B	30 12 50	J A 40 years	6	″	30 back	—	35	″
Samoa A	12 1 51	J 1½ years	7	Yaws	21 back	32	3	No
Chicago	9 2 51	W McD 25 *years*	8	Syphilis	11 back	—	55	Yes
Indonesia B	3 3 51	S 11 years	9	Generalized yaws	5 back	—	24	″
Haiti A	7 3 51	M E 9 years	10	Generalized yaws	75	—	27	″
Haiti B	7 3 51	J L S 11 years	11	″	70	—	39	″
Iraq A	29 4 52	S 7 years	12	Bejel	69	30	37	″
Mexico A	13 1 53	G A 18 years	13	*Primary syphilis*	—	22, 28, 35 [b]	9	″
Samoa D	24 1 53	I 1 year	14	*Generalized* yaws	—	13, 12, 10	15	″
Samoa E	24 1 53	M 3 years	15	″	—	15, 10, 10	13	″
Samoa F	24 1 53	M 4 years	16	″	—	47, 24, 30	14	″
Bechuanaland C	3 4 54	G G 22 *years*	17	Non venereal treponematosis	—	52	8	″
Bechuanaland D	4 4 54	M M 5 *years*	18	″	—	23	7	″
Gambia A	9 7 55	B K 4 years	19		—	40	2	″
Gambia B	9 7 55	S S 11 years	20		—	37	2	″
Gambia C	9 7 55	T S 4 years	21	″	—	46	2	″
Gambia D	9 7 55	O G 4 years	22	″	—	43	2	″

[a] As of 1 September 1955
[b] No lesions—lymph nodes darkfield positive on indicated day

Comparative data on the incubation period of the disease in the first-passage animals are also of limited value because of the differing circumstances under which the isolations were made: some in the relatively high environmental temperature of the tropics, others in the rather cool temperature of the temperate zone, and still others partly under both conditions Moreover, inocula from different sources probably varied considerably in the number of treponemes injected For what they are worth, however, the data on initial incubation periods following intratesticular inoculation of rabbits are given

Of 31 syphilis strains the shortest incubation period was 14 days, the longest 82, the median being 40 days. Of 11 yaws strains the shortest incubation period was 28 days, the longest 75, the median being 52 days Three strains of bejel treponemes were isolated by intratesticular inoculation of rabbits with incubation periods of 53, 67, and 69 days, respectively The two strains of so-called endemic syphilis isolated in rabbits had incubation periods of 60 and 70 days, respectively. Most of the strains isolated in the past three years were initially inoculated into hamsters rather than rabbits, and data for these are not included in the foregoing analysis

Inoculation of hamsters

With the demonstration in 1949 by Geiman & McKee [8] that the hamster was susceptible to yaws as well as to syphilis treponemes, this laboratory animal was utilized either alone or in conjunction with the rabbit for subsequent isolations However, not until 1952 was it appreciated that hamsters infected with one of the treponemal group of organisms customarily contain numerous treponemes in their lymph nodes even when no clinically discernible lesion is present, and undoubtedly some of the initial transfers into hamsters were erroneously regarded as unsuccessful prior to that date. Eight strains have been isolated since 1952 solely in hamsters (Table Ic).

Unsuccessful isolations

During the course of these studies 76 different strains of treponemal organisms have been successfully isolated in laboratory animals. In the same period transfers of material, known to contain treponemal organisms from 10 patients with syphilis and 17 patients with yaws, for one reason or another failed to result in successful isolations Taken at their face value, however, these figures may be misleading, for in many instances failure was associated with secondary bacterial contamination in a situation in which transfer had been made to one animal only, a practice not uncommon where patients with yaws and syphilis were numerous and rabbits rather scarce Following inoculation of human material into the testis of rabbits there is often initially a slight non-specific reaction manifested as a palpable

been shown, contrary to what might be supposed, that the monkey (*Macacus rhesus*) is a less favorable animal for initial isolation of yaws strains than the rabbit, and subsequent experience in this laboratory is in agreement with that observation (See Chapter 2.)

Inoculations were commonly made into the body of one or both testes of the rabbit with amounts up to 1 0 ml, preferably less. A small amount (0 1 ml) was often injected intradermally into one or both scrota. Intracutaneous inoculation on the clipped back of the animal was occasionally employed, and yielded positive results in some cases Inoculation on to the surface of a granulating wound, as described by Chesney, Turner & Halley,[5] was used in 63 instances with positive results in only 3.

During the course of experiments on the possible transmission of yaws by *Hippelates* flies,[8] rabbits were infected by this means In one type of experiment the animal was infected by flies feeding first on an infectious yaws lesion of man and then upon a granulating wound or a freshly scarified area on the scrotum, of 68 rabbits on which supposedly infected flies were permitted to feed, specific yaws lesions developed in the wound in 7 In another type of experiment, the esophageal diverticula of *Hippelates* flies which had fed on infectious yaws lesions were dissected out at various intervals after feeding and inoculated into wounds of the back or scrotum Of 28 rabbits so exposed, 8 showed definite evidences of infection. Incubation periods in the rabbits ranged from 16 to 87 days, with all but 3 being between 26 and 45 days These experiments have been previously reported by Kumm, Turner & Peat.[9]

The evolution of disease phenomena in the inoculated rabbits resembled that seen after the inoculation of adapted strains, except that on the whole the lesions in the first animal passage tended to be smaller and more evanescent. This was particularly true of the lesions produced initially by the yaws strains Often the only lesion detected was slight enlargement of the head of the epididymis on the inoculated side, the time for the development of this type of lesion, frequently over 50 days, suggests that it is a metastatic lesion rather than the initial focus

As a group, syphilis strains tended to have shorter incubation periods than yaws strains. While the difference in incubation periods appears to be a significant one, it must be borne in mind that the syphilis patients as a group were in a substantially earlier phase in the evolution of their disease than were most of the yaws patients. To what extent this factor influenced the incubation period in the initial transfer, it is difficult to say. Moreover, yaws lesions in rabbits are as a rule much smaller and more difficult to detect, so that this factor may have delayed recognition of the changes in the rabbit's testis But whatever the explanation for the differences observed in incubation period, these same differences do not appear to be restricted to the initial transfers, and were commonly noted also in many subsequent passages

Altogether 8 hamsters were inoculated from the 3 positive hamsters, and 4 blind passages were made into another generation with no evidence of disease in these 12 animals.

The known variation among species of syphilis, yaws, and cuniculi treponemes in their susceptibility for humans, rabbits, and hamsters, respectively, may be an argument that strains or species of treponemes may exist to which laboratory animals are not susceptible. On the other hand, the demonstration of treponemes in 3 animals inoculated from 3 different pinta patients suggests that hamsters, at least, are not wholly resistant.

Another explanation for the failures may lie in the fact that human infections of relatively long standing were used for transfer. The presence of specific antibody in this material may greatly decrease the infectivity, particularly when the inoculation is made into a new animal species. It is perhaps pertinent that León-Blanco,[11] who reported a single successful transmission from a case of Cuban pinta into a rabbit, first inoculated the material into a human volunteer, and subsequently inoculated the rabbit from the early primary lesion of the human volunteer.

* * *

With the reservation that our experience in the isolation of strains is far from a controlled experiment, and that it is necessary to rely on impressions only, it appears that both the rabbit and the hamster are satisfactory animals for the isolation of most new strains of treponemes, and indeed there are indications that of these two the hamster is the more satisfactory, particularly for isolation of the yaws type of organism.

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infiltration, or even an actual nodule, in the body of the testis, but in the presence of substantial numbers of contaminating bacteria, an abscess may form, under which circumstances there will be much less chance of a successful isolation

Of course, any of the other factors such as temperature, antibiotics, or prior cuniculi infection, which are known to influence treponeme infections (see Chapter 3), may adversely affect the chances of a successful isolation, and it is perhaps surprising that more failures have not been encountered, particularly in animals shipped long distances after inoculation.

Attempted isolation of pinta treponemes

Although the same methods were employed in attempting to isolate pinta strains, we have been unable to establish a strain in animals. Three of 30 hamsters, however, when examined 10 weeks after the inoculation of pinta material, contained actively motile treponemes in their inguinal lymph nodes as follows:

(1) H-146, a male hamster was inoculated with material from A S, a 15-year-old male Indian, by Drs D H Hollander, J. Olarte and G Varela, 17 January 1952. When the animal was sacrificed after 73 days, no symptoms having been present in the meantime, the apparently normal inguinal lymph node contained 2 typical treponemes identified in the emulsion with 0.1 ml of serum saline by 3 observers.

(2) H-154, a male hamster was inoculated with material from M R, a 42-year-old male Indian in Mexcala, Guerrera, Mexico, 19 January 1952. A scaly area noted in one groin after 4 weeks was repeatedly darkfield negative When the animal was sacrificed 67 days after inoculation one inguinal node appeared definitely enlarged An emulsion in 0 1 ml of serum saline contained 3 typical treponemes identified by 3 observers. This and the first hamster were part of a group of 8 hamsters and 2 rabbits shipped from Mexico City to Baltimore at the same time; 3 hamsters did not survive, all animals remained asymptomatic, and the 3 other hamsters had darkfield-negative lymph nodes.

(3) H-439, a male hamster was inoculated with material from J. H, an 18-year-old male Indian in Iguala, Guerrera, Mexico by Dr J. Olarte, 15 January 1953. When the hamster was sacrificed after 72 days without lesions the inguinal nodes were enlarged. Emulsion of one node in 0.1 ml of saline contained 2 treponemes identified by 4 observers. Two other hamsters inoculated from the same patient and 6 from another pinta patient included in the same shipment were negative, while 3 hamsters inoculated with Mexico A, a strain of syphilis which was also in this shipment, were each positive. In two other shipments 15 hamsters were inoculated from pinta patients without further success.

Chapter 2

THE EXPERIMENTAL DISEASE IN LABORATORY ANIMALS

To a certain extent treponemal infections in laboratory animals have common clinical and histopathologic features These features are well known for some animal species and for some species of treponemes, and it is not our intention to duplicate reports on this aspect of the subject; but together with a presentation of studies from this laboratory, some of which have not hitherto been reported, the general features and the evolution of experimental treponemal infection will be described.

The rabbit, first successfully inoculated by Bertarelli [3] in 1906, has been the principal laboratory animal for the study of treponemes in recent years The usual course of experimental syphilis in the rabbit has been described in great detail by Brown & Pearce [11, 12] It should be noted, however, that the classic experiments of Brown & Pearce with rabbits, as well as those of Neisser [48] and his associates, and Schöbl [58] and his associates with monkeys, were carried out at a time when procedures such as the treponemal immobilization (TPI) test and the treponemal agglutination (TPA) test were not yet available In addition, many of the factors which are now known to interact in the progress of experimental infection were still unappreciated Much of the improvement of contemporary methods over the older observations may be attributed to increased knowledge in these fields

In this laboratory also, the rabbit has received particular attention Studies have been directed, first, toward improvement of the experimental methods, and, second, toward clarification of the specific changes which the infection induces, together with examination of the factors which influence the course of the infection These two objectives are interlocked, for improvement in the method permits a more precise measurement of a given variable factor, and this then allows the adjustment of the variable factor at its optimum level which, in turn, increases the precision of the method. Major improvements in the quantitation of infection have been the utilization of intracutaneous pattern inoculation, and the use of the incubation period as an index of the size of the inoculum

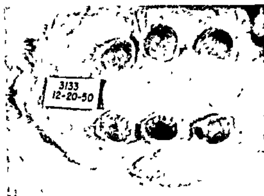
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PLATE I MACROSCOPIC LESIONS

A



B



The monkey, the first animal to be infected successfully with treponemes by Metchnikoff & Roux [46] in 1903, has furnished the basic pattern of experimental animal infection through the studies of Neisser [45] and his associates on syphilis, and those of Schöbl [58] and his associates on yaws. In this laboratory studies were conducted in 1946 and 1947 by the senior author and his associates, at that time Dr Elaine L. Updyke and Dr Mary Cumberland Yurchenco. These studies were planned to determine whether *T cuniculi* infection might have some potentiality as an immunization procedure against syphilis in primates, a notion which has some scientific validity in view of the demonstrable cross-immunity between the two infections in rabbits (See Chapter 7)

Guinea-pigs, rats and mice are known to be susceptible to treponemal infection, mice, in particular, have often been studied as experimental hosts, but these animals have the great disadvantage that the infection does not ordinarily produce a tissue response which is macroscopically observable.

Hamsters, however, seem to differ in two respects which make them more advantageous for use in experimental treponemal infections. After inoculation with treponemes, their lymph nodes often contain large numbers of treponemes,[5, 6, 8] and following inoculation of *T. pertenue* they often develop extensive chronic skin lesions.[20] In addition, when compared with the rabbit they have the advantage that no known treponemal infection occurs naturally in them. Moreover, as indicated by the observations reported here, new information on the host reactions induced by various strains of treponemes can be obtained by study of the infection in hamsters.

DESCRIPTION OF PLATE I

A. CHARACTERISTIC INTRADERMAL SYPHILOMAS OF RABBIT (MACROSCOPIC APPEARANCE)

Photograph of back of rabbit taken 26 days after the intradermal inoculation of approximately 50 000 treponemes (Nichols strain) at each of the 6 sites Lesions appeared on the 9th day

Reproduced from Shwartzman, G, ed (1953) *The effect of ACTH and cortisone upon infection and resistance*, New York, by kind permission of the Columbia University Press

B. INTRADERMAL SYPHILOMAS IN CORTISONE-TREATED RABBITS (MACROSCOPIC APPEARANCE)

Photograph of a rabbit inoculated in the same manner and at the same time as the animal illustrated in (A) Lesions appeared on the 9th day Injections of cortisone (3 mg/kg body-weight) were given twice daily, beginning on the 3rd day after inoculation Lesions of this character were observed in 13 of the 14 animals in the group which had received cortisone treatment Note the globoid shape of the lesions, the relative pallor, and the absence of ulceration

Reproduced from Shwartzman, G, ed (1953) *The effect of ACTH and cortisone upon infection and resistance*, New York, by kind permission of the Columbia University Press

Treponemal Infection of the Rabbit

Clinical course of syphilis

The development of experimental treponemal lesions can be observed most readily after the inoculation of syphilis treponemes on to the shaved surfaces of rabbits' backs When conditions are suitable, after an incubation period which may vary from a few days to several weeks, depending on the size of the inoculum, there appears a small papule which increases rapidly in size for about ten days At this stage the lesion is characteristically elevated, with a flat surface and a circumscribed sloping base, and may measure as much as 10-15 mm in diameter

Following this phase of active progression, an alteration in the character and the development of the lesion occurs—sometimes abruptly but more often insidiously after a more or less prolonged interval Presumably along with specific immunologic changes occurring in the host, the lesion begins to become more indurated, and necrosis and ulceration appear

The back of a rabbit with lesions in this stage is shown in Plate I (A) (facing page 32) This animal, an adult male albino, from a group of 12 untreated controls in a large-scale cortisone experiment, was inoculated intradermally at 6 sites on the shaved surface of the back with an emulsion of syphilitic rabbit testis containing approximately 50 000 treponemes per injection site On the 9th day tiny papules were noted, on the 19th day they were described as typical indurated papules with broad bases, on the 22nd day some discoloration of the surface appeared, and by the 26th day, at the time the photograph was taken, this had progressed to frank necrosis and ulceration

Subsequently the lesion may regress and heal, with or without some residual scarring, or there may be continued activity for several weeks, especially at the margin of the lesion Rarely, complete healing may occur in the central portion while the periphery shows continuing signs of activity Ordinarily the complete cycle to healing requires at least a month.

In any large series of rabbits inoculated with aliquots of the same treponemal suspension, in most cases the lesions will regress in a uniform manner, but in an occasional animal the lesions will persist much longer than in the others No clear-cut differences in measurable antibody have been noted as a possible basis for this seemingly erratic behavior on the part of an occasional experimental rabbit host

Generalized lesions

Following the inoculation of treponemes, there occurs a rapid dissemination from the sight of inoculation For example, Raiziss & Severac [31] recovered *T pallidum* from the general blood stream of rabbits within five

All strains of syphilis spirochetes studied appear to have the capacity to invoke skin and bone lesions in rabbits, but considerable variation has been observed from strain to strain in this respect.

On the other hand, yaws strains appear to have much less tendency to induce generalized lesions in rabbits.[41, 66] With regard to the strains of bejel and endemic syphilis, and the strains causing related syndromes that have been studied in this laboratory, adequate observations have not been made, owing to the large number of animals that would have had to be maintained over long periods Suffice it to say that generalized skin and bone lesions have not been observed in animals inoculated with these strains.

After the initial lesions and the generalized lesions have subsided, no external evidence of infection remains, yet rabbits continue to harbor virulent treponemes for very long periods Strains of syphilis have repeatedly been recovered from rabbit lymph nodes after periods of several years Frazier, Bensel & Keuper[18, 19] have found the blood of some rabbits to be infective more than three years after the disease had become asymptomatic. It appears that animals do not have the capacity to effect a biologic cure in experimental syphilis without the help of therapy, the situation with respect to yaws and cuniculi infections is less certain

Histologic features of syphilis in rabbits

The histologic picture of a syphilitic lesion is an admixture of several responses which almost always overlap, and which may be individually inconspicuous in any single lesion It is convenient to distinguish three stages in the total reaction (*a*) the change produced by the accumulation of treponemes and characterized by the presence of a mucoid material, (*b*) the changes consequent upon the immune reaction and characterized by an infiltration with mononuclear cells, and (*c*) changes associated with necrosis followed by the familiar processes of inflammation and repair.

Mucoid material in syphilis lesions The first reaction both in point of time and in importance, because of its probable origin from the treponeme itself, is the accumulation of a mucoid substance at the site of inoculation

The presence of mucinous material in syphilitic lesions was early noted by Uhlenhuth & Mulzer[69] and by Graetz & Delbanco.[21, 22] Akatsu[1] in Matsumoto's[41] laboratory also noted that mucoid or a " myxomatous change " was a characteristic finding in the actively proliferating stage of experimental rabbit syphilis Scott & Dammin[59, 61] observed that the metachromatic staining property of the material was similar to that described for hyaluronic acid by Strempel & Armuzzi[62] and Gregoriew & Jarisheva[23] Turner & Hollander[67, 68] reported that the metachromatic staining of this material was abolished by treatment with bull-testis hyaluronidase Scott & Dammin[59, 61] reported that the metachromatic staining could be abolished by pre-treatment of the tissue with testicular hyaluronidase, but not by

minutes after intratesticular inoculation, and numerous investigators have made such recovery within a period of several hours. Generalization may perhaps be facilitated by the disruption of capillaries and small venous channels due to the force with which the material is injected. There can be no question, however, that *T. pallidum* and presumably other species of treponemes have an inherent capacity to extend beyond the site of initial contact, irrespective of the factor of mechanical dissection of lymphatic or vascular channels. Mahoney & Bryant,[42, 43] for example, noted that *T. pallidum* applied to the intact mucous membrane of rabbits could be demonstrated after two hours in the deeper layers of tissue, and many investigators have noted generalized infection after similar methods of inoculation[15]

The site of inoculation appears to influence the extent and distribution of generalized lesions Chesney & Schipper[14] compared the incidence of generalized lesions in a large series of rabbits inoculated identically by intracutaneous, intratesticular, or intravenous routes Five out of 28 rabbits inoculated intracutaneously; 14 out of 27 inoculated intratesticularly, and 28 out of 29 inoculated intravenously developed generalized lesions.

Following intratesticular inoculation of one testis, the opposite testis becomes involved in a high proportion of rabbits, although the lesion in the second testis rarely reaches the size of that of the initial orchitis In a smaller proportion of animals generalized lesions of the skin and sometimes of the bones develop. The skin lesions vary from erythematous macules to well developed papules, probably in many animals minimal macular lesions may go unrecognized. As will be pointed out in Chapter 3, skin lesions tend to develop in peripheral areas where the surface temperature is somewhat lower than the general body temperature of rabbits Thus, these lesions are most frequently observed on the lower fore and hind legs, at the base of the ears, about the nose and along the tail.

Bone lesions follow much the same pattern as skin lesions as regards location and timing Lesions occur most frequently along the bones of the foreleg, the metatarsals, the nose, and the tail The lesion is predominantly a periostitis, less often there is invasion of the bone, occasionally severe enough to lead to pathological fracture (Chesney & Turner, unpublished observations, 1931) Good descriptions of both bone and skin lesions have been given by Brown & Pearce[11, 12] and by Matsumoto[44]

Metastatic lesions occur usually between 35 and 60 days after inoculation. The time relationship in the evolution of the disease process may be accounted for, first, by the lapse of time from original inoculation of the animal to generalization, and, second, by the time required for the mul-

host.

stimulus, and to arise in association with areas of necrosis, or ulceration and secondary infection The necrosis is not seen in small lesions, but only in extensive lesions, where it may be a mechanical result of failure of the oxygen supply from compression of the small vessels. It should be noted that tissue cells and treponemes have dissimilar oxygen requirements, from which it can be inferred that conditions favorable for optimum growth of treponemes do not coincide with conditions favorable for optimum growth of tissue cells

The prototype of human gummatous tertiary lesions has not been produced in animals, except possibly in a few instances in monkeys[58] Presumably this reaction of necrosis in a "hypersensitive" or "allergic" host is of a different character from that commonly observed in experimental lesions in rabbits

Many of the usual histologic features of syphilis can be seen in a characteristic lesion such as that illustrated in Plate II (facing page 40), which was taken from a companion rabbit (No 31-72) to that illustrated in Plate I (A) This animal presented an identical history of infection except that ulceration started a few days later When the lesion was excised for microscopic examination 26 days after inoculation and 17 days after the first appearance of the lesion it was in a slightly earlier stage than the lesion of the rabbit No 31-66 illustrated in Plate I (A) At this time darkfield examination of scrapings from another lesion in the same rabbit showed many treponemes The entire skin thickness was fixed in formaldehyde solution, and paraffin sections were prepared in the usual manner A hematoxylin- and eosin-stained section is shown at a low magnification in Plate II (A)

The section shows an oval nodule lying between the epithelum above and the muscle layer below It is chiefly composed of lobular areas which are not stained by either hematoxylin or eosin The structures of the skin including the hair follicles and even individual collagen fibers are scattered through the unstained areas as if some material had diffused and infiltrated between the individual cells and fibers of the lesion However, a few connective-tissue septa containing small blood vessels seem to have resisted the process and formed lines of cleavage, as if to separate the lesions into lobules

Under higher magnification even the very clear areas are seen to be traversed by a regular network of cells and fibers, superficially resembling fat septa in paraffin sections, but while fat is represented by empty spaces from which something has been dissolved, in this typical syphilitic lesion the clear areas actually contain a mucoid material which is demonstrable by appropriate stains A section from the same block stained with toluidine blue (Plate II (B)) shows that these areas contain an amorphous material with a distinctly purplish color contrasting sharply with the azure blue of the inflammatory cells and the tissue cells in the section. Another section—also from the same block—stained with toluidine blue after pre-treatment with

treatment with streptococcal hyaluronidase. This suggests that the material may be a chondroitin sulfate rather than hyaluronic acid, according to the studies of Meyer & Rapport,[47] who state that chondroitin sulfate A found in cartilage is hydrolyzed by testicular but not by pneumococcal hyaluronidase.

Turner & Hollander [67, 68] have suggested that the mucoid material is actually a product of the treponeme, in the nature of a capsular substance or a slime layer excreted by the treponeme This is a view contrary to that held in the past, when it has been regarded as altered connective tissue. Scott & Dammin,[59-61] for example, designated it " embryonal connective tissue ", and Akatsu [1] called it a " myxomatous change "

While the evidence that the mucoid material is a product of the treponeme itself is largely circumstantial, we believe it is incontestable. It is a familiar fact that many micro-organisms produce complex polysaccharides, some even producing hyaluronic acid, and it is also perhaps relevant that Noguchi [49] described a small dental treponeme which produced " mucin " *in vitro* But most convincing is the observation, made during the cortisone experiments described in Chapter 3, that the accumulation of mucoid material parallels an increase in the number of treponemes at a time when the cellular reaction is held in restraint by the action of cortisone.

Ikegami [28] and Matsumoto [41] reported that the " myxomatous tissue " is also present in the experimental lesions produced in rabbits by yaws treponemes It should be noted that the mucoid material is prominent only in the early stages of rapidly progressing lesions. It was not a feature of earlier histologic studies of syphilis in rabbits reported by Rich, Chesney & Turner,[52] or of yaws in rabbits reported by Ferris & Turner.[17] In general little or no mucoid material accumulates in yaws or cuniculi lesions as compared with syphilis lesions. Further discussion of this subject will be found in connexion with the cortisone studies in Chapter 3, and also in Chapter 7

Cellular reactions in lesions of syphilis When the syphilitic lesion has reached its peak, there occurs a massive infiltration with mononuclear cells, most of which are lymphocytes, while others resemble epithelioid cells. The lymphocytic infiltrate we interpret as the principal expression of the specific immune response of the host The epithelioid reaction may also be a part of this response, however, it is much less prominent in treponemal lesions than in certain others In tuberculosis, it has been correlated with the special waxy substance of the tubercle bacillus—a material which has no known counterpart in the treponeme.

Many lesions also contain polymorphonuclear leukocytes, or, more precisely in the rabbit, the " pseudo-eosinophile ". Unlike the mononuclear cells, which are considered to be the specific response to the treponeme, the polymorphonuclear cells are believed to be unrelated to the treponeme

But, as described in Chapter 7, later passages of one of the strains in question (YD) showed a progressive loss of this feature, to the degree that it now resembles strains of syphilis much more than those of yaws

Matsumoto [41] and his co-workers, who have also been interested in the comparative features of yaws and syphilis in the rabbit, concluded that the granular periorchitis was one of the most characteristic changes in yaws after either intratesticular or intravenous inoculation Matsumoto [41] and Ikegami [28] noted the characteristic "myxomatous tissue" in these granular lesions While we have not examined sections specifically prepared to clear up this point, it is believed that the difference between these lesions and the more widespread testicular lesion commonly observed in syphilis is a quantitative one, reflecting the areas which are suitable for the production of lesions, and the relative quantities of hyaluronic acid produced by the two strains, rather than a qualitative difference in response On the other hand some sections from testes exhibiting these lesions have shown tiny areas of necrosis within the granules, suggesting that these are miliary gummata—a response qualitatively different from that which is characteristically seen in early rabbit or human lesions

It is of interest that Jahnel & Lange [31] isolated a strain of yaws in Sumatra which did not show the granular periorchitis, and among the strains of syphilis isolated at the International Treponematosis Laboratory Center, two (WM and Mexico) frequently produced this lesion This will be further discussed in Chapter 7

Cuniculi infection in rabbits

Observations on the course of cuniculi infection in rabbits, together with a review of the literature on the subject, have been reported from this laboratory by McLeod & Turner [40] The lesions following naturally acquired infection in rabbits are characteristically described as slightly elevated scaly patches, or eroded sores covered by a scab, which bleed readily upon slight scarification They show little or no induration and may be distinguished without difficulty from the lesions of experimental syphilis Naturally occurring lesions produced by *T cuniculi* are usually seen about the prepuce, vagina, anus or scrotum, and less frequently on the nose, eyelids, lips and paws.

Following induction of the experimental disease by intratesticular inoculation, a rather characteristic lesion develops in about half the inoculated animals This lesion consists of fine granular nodules scattered throughout the parietal layer of the tunica vaginalis, often with no macroscopically recognizable lesions in the visceral layer of the tunic or in the body of the testis These lesions are rich in treponemes The characteristic lesion described above may give the impression on palpation that the body of the testis is either slightly firm or finely granular. Occasionally, however,

bull-testis hyaluronidase (Plate II (C)) does not show any trace of the purple metachromatic stain The same areas which stained metachromatically with toluidine blue are unstained both in the section pre-treated with hyaluronidase and in the hematoxylin and eosin preparation.

About the periphery of the lobules and extending along the connective-tissue septa surrounding some of the blood vessels and hair follicles, there are accumulations of mononuclear cells These cells are chiefly lymphocytes At one end of the lesion in the largest mass of cells (see Plate II) there are also sheets of larger cells, presumably young epithelioid cells but also resembling young connective-tissue cells, with large clear nuclei.

Scattered throughout the nodule are many pseudo-eosinophiles. The lesion illustrates the relation of these cells to necrosis of tissue. There is a zone of definite necrosis extending about 1 mm in depth from the surface of the lesion, with a sharp boundary containing very large numbers of pseudo-eosinophiles and nuclear remnants of destroyed cells An extensive *diffuse necrosis is also present which is not apparent either in the gross or in* the low-power magnification When examined at higher magnification, however, karyorrhexis and accumulation of pseudo-eosinophiles are seen about the connective-tissue septa and between the lobules of mucoid material in areas throughout the lesion

Distinctive features of yaws in rabbits

The different behavior of various strains of treponemes in animals will be the subject of later chapters (Chapters 7, 8 and 9) For the present it will be sufficient to note that the foregoing account of a syphilis lesion in rabbits is not descriptive of the behavior of all strains of treponemes in rabbits. In general, strains of syphilis act in this manner, although even among syphilis strains there appears to be some gradation of virulence for the rabbit On the other hand most yaws strains appear to produce much less reaction at the site of inoculation and have much less tendency to produce disseminated lesions. Nevertheless there is nothing which serves to distinguish absolutely the individual syphilis lesion from the individual yaws lesion,[17] with the possible exception of the testicular lesion, which has been designated granular periorchitis.

Pearce & Brown [50] first called attention to the fact that yaws strains characteristically produced tiny miliary nodules on the tunic of an inoculated rabbit testis, while the interior of the testis was relatively little involved. This is quite dissimilar to the usual appearance of syphilis in the rabbit testis, which caracteristically involves the interior, without provoking, at least in the early stages, any change in the tunic.

The eight strains isolated by Turner & Chambers [65] in Haiti in 1929-30, when studied by Turner & Chesney,[66] regularly exhibited this lesion, as did the Jamaica strains studied by McLeod & Turner.[40]

PLATE II MICROSCOPIC FEATURES

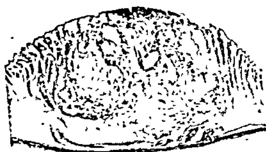
A



D



B



E



C



F



DESCRIPTION OF PLATE II

A. INTRADERMAL SYPHILOMA OF RABBIT (MICROSCOPIC APPEARANCE): HEMATOXYLIN AND EOSIN STAIN

Microphotograph (×7) of a cutaneous lesion excised from a companion rabbit to that illustrated in Plate I (A). Approximately 50 000 treponemes (Nichols strain) were inoculated. Lesions appeared on the 9th day. A biopsy of full skin thickness was taken on the 26th day, fixed in neutral formaldehyde solution, embedded in paraffin and stained with hematoxylin and eosin. See descriptive comments in Chapter 2, page 37

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B. INTRADERMAL SYPHILOMA OF RABBIT (MICROSCOPIC APPEARANCE): TOLUIDINE-BLUE STAIN

Microphotograph (×7) of a duplicate section from the same block as (A); stained with toluidine blue. Note the purplish metachromatic staining of the areas within the lesion which are unstained by hematoxylin and eosin

C. INTRADERMAL SYPHILOMA OF RABBIT (MICROSCOPIC APPEARANCE): TOLUIDINE-BLUE STAIN AFTER TREATMENT WITH HYALURONIDASE

Microphotograph (×7) of a duplicate section from the same block as (A) and (B); treated with bull-testis hyaluronidase and then stained simultaneously with (B). Note the complete abolition of the metachromatic staining

D. INTRADERMAL SYPHILOMA IN CORTISONE-TREATED RABBIT (MICROSCOPIC APPEARANCE): HEMATOXYLIN AND EOSIN STAIN

[illegible]

descriptive comments in Chapter 3, pages 83-84

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E. INTRADERMAL SYPHILOMA IN CORTISONE-TREATED RABBIT (MICROSCOPIC APPEARANCE): TOLUIDINE-BLUE STAIN

Microphotograph (×7) of a duplicate section from the same block as (D); stained with toluidine blue. Note the purplish metachromatic staining of the areas within the lesion which are unstained by hematoxylin and eosin

F. INTRADERMAL SYPHILOMA IN CORTISONE-TREATED RABBIT (MICROSCOPIC APPEARANCE): TOLUIDINE-BLUE STAIN AFTER TREATMENT WITH HYALURONIDASE

Microphotograph (×7) of a duplicate section from the same block as (D) and (E); treated with bull-testis hyaluronidase and then stained simultaneously with (E). Note the complete abolition of the metachromatic staining

firm testicular lesions develop which resemble those seen in syphilis. Although the degree of induration tends to increase with continued passage of a strain, the extreme induration and tremendous enlargement of the testis, so characteristically induced by many strains of syphilis treponemes is rarely, if ever, observed Regardless of the type of initial lesion in the testis, nearly all rabbits observed for two months or longer develop indurated nodules in the head of the epididymis.

When only one testis is inoculated, involvement of the other testis frequently follows The metastatic testicular lesions are often granular, like the initial lesion, but are commonly less extensive Extensive generalized lesions of the skin, nose, mouth, eyes, ears and paws occur in a substantial proportion of rabbits observed for 2-4 months after initial inoculation in the testis These lesions differ in character from those observed in experimental syphilis, in that the former are flat, with a scaly surface and little or no induration Lymph nodes of infected rabbits usually remain infective for normal rabbits for long periods after initial inoculation. Standard serological tests, TPI tests and TPA tests become positive during the course of the disease; the serological pattern does not seem to differ significantly from that observed in experimental syphilis and yaws. The experimental disease responds well to drugs which usually have an antitreponemal action The question of immunity will be considered in Chapters 7 and 8

Methods of quantitation

Intradermal pattern inoculation A considerable degree of accuracy has been attained in the measurement of syphilis infection by the technique of intradermal inoculations of known numbers of treponemes at multiple sites on rabbits' backs This was first described by the senior author in 1939 [81] in a study in which neutralizing or "protective" antibodies were demonstrated by this technique

In this early study intracutaneous inoculations were made at 6 sites The advantages derived from the multiple inoculations were stated as follows

"If syphilitic lesions develop at each site of an area, a characteristic pattern is noted. This pattern assumes importance when the lesions begin to develop, for it aids materially in distinguishing between syphilitic lesions and non-specific lesions, which are not infrequently encountered The pattern of beginning syphilitic lesions, is unmistakable, however, and greatly facilitates establishing the incubation period of the lesions"

The size of inoculum and its relation to the incubation period It is convenient, and perhaps not illogical, to consider treponemal infections in terms of two opposing trends, one the growth and multiplication of treponemes and the other their death and destruction This picture is, of course,

In the study reported by Cumberland & Turner,[18] at various intervals after inoculation of a measured inoculum the testes were removed and cut into 6 equal transverse sections. A loopful of 10% serum-saline was pressed gently but firmly into the surface of each section and between 0 0025 ml and 0 005 ml of the fluid was collected on a coverslip and the number of treponemes counted in 50 oil immersion fields in the darkfield The sum of the treponemes in the 6 preparations was taken as an index of the population of the whole testes The results are shown in Table II and are plotted

TABLE II NUMBER OF TREPONEMES OBSERVED IN PREPARATIONS OF RABBIT TESTES AT VARIOUS INTERVALS AFTER THE INOCULATION OF DIFFERENT QUANTITIES OF TREPONEMES *

Time	Inoculum					
	10 000 000		1 000 000		100 000	
	Number of testes	Number of treponemes (median)	Number of testes	Number of treponemes (median)	Number of testes	Number of treponemes (median)
0	14	81	6	7	4	1
2 hours	16	6				
1 day	18	5	4	1		
3 days	15	23	4	2		
5 days	12	46	4	8	4	1
7 days	10	246	4	25	4	2
9 days					4	5
11 days	4	2 998	4	62	6	17
13 days			6	152	4	44

* Adapted from Cumberland & Turner[18]

on a logarithmic scale in Fig 1 It is seen that there was a constant logarithmic increase beginning within 24 hours after inoculation The organisms apparently increased at a constant rate regardless of whether the initial inoculum consisted of 10 000 000, 1 000 000, or 100 000 treponemes It was calculated that an average of 108 hours or 4 5 days was required for a tenfold increase in the number of spirochetes This is equivalent to an average of 33 hours for a single division, on the assumption that each spirochete divides into two.

Hollander & Turner[28] obtained similar figures from a series of testes examined at intervals during the incubation period after inoculation of 100 000 treponemes. The testes were cut into 8 or 10 pieces, shaken in

enormously complicated by development of immunity within the host, accompanied by increasing ability of the host to destroy treponemes, and a changing qualitative response to the products of this destruction However, the immune response develops slowly, and is detectable and measurable only after a very large number of treponemes have accumulated. The early part of the infection, therefore, including the incubation period and a short time thereafter, is not appreciably influenced by immunity, and the behavior of the treponemes during this period is determined chiefly by the forces which influence their growth and multiplication. During this phase of the infection the animal approaches an inert culture medium, and the growth of the treponemes approximates that of micro-organisms during the logarithmic growth phase *in vitro* Several circumstances make this separation between the early and the later stages of treponemal infections more precise with treponemes than it is with many other infective agents. The organisms grow relatively slowly, the natural defenses or mechanisms of resistance of the host seem to play a minor role with this infection, and the production of the specific antibodies proceeds relatively slowly.

In the earliest investigations of experimental syphilis it was suspected that shorter incubation periods follow larger inocula. Chesney & Kemp [13] in 1925 established this by inoculating serial dilutions of the same emulsion Magnuson, Eagle & Fleischman [41] reported similar observations in experiments in which the *number of treponemes injected was determined* by the enumeration of treponemes in measured drops Magnuson calculated that his observations were in accord with the assumption that a steady logarithmic increase of treponemes occurs during the incubation period at the rate of one division about every 30 hours.

All the evidence indicates that the incubation period is in no sense a latent period, but is rather a time which is required for the treponemes to multiply and increase to the extent that a palpable or visible mass is induced which can be recognized as a lesion Since this mass accumulates sooner when the original inoculum is large, the incubation period varies inversely with the size of the inoculum Under suitable conditions treponemes divide at the rate of about once every 30-33 hours and increase roughly tenfold each 4 days Clinically recognizable lesions begin to appear when the treponeme count in a local area reaches the order of 10 000 000 treponemes With very large inoculations of the order of 5 000 000 organisms or more, the incubation period is about 2 or 3 days and may be difficult to distinguish *from a transient local reaction to the inoculation.*

There is ample experimental evidence that during the incubation period the inoculum is increasing logarithmically. Rich, Chesney & Turner [52] detected increasing histologic changes at the injection sites long before the gross lesions appeared, and Cumberland & Turner [16] found a logarithmic progression in the number of treponemes by direct counts during the incubation period

TABLE III NUMBER OF TREPONEMES EXTRACTED FROM NORMAL RABBIT TESTES AT VARIOUS INTERVALS AFTER INTRATESTICULAR INOCULATION OF 100 000 ORGANISMS *

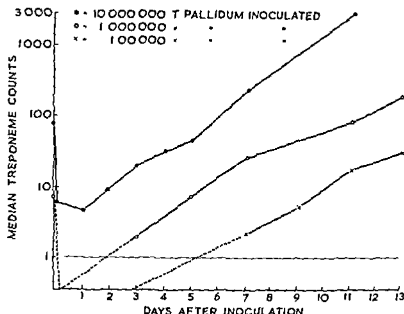
Day	Experiment E		Experiment G		Experiment H	
	Rabbit testis	Number of treponemes	Rabbit testis	Number of treponemes	Rabbit testis	Number of treponemes
0	2766 R	0				
0	2766 L	0				
4	2773 R	0				
4	2773 R	0				
8	2767 R	2 000				
8	2767 L	2 000				
12	2772 R	500 000	2899 R	2 000 000	4570 R	1 700 000
12	2772 L	200 000			4570 L	2 400 000
14			2900 R	8 000 000	4571 R	5 000 000
14					4571 L	3 500 000
16	2768 R	7 000 000	2902 R	18 000 000	4572 R	11 750 000
16	2768 L	3 000 000			4572 L	3 750 000
18			2903 R	54 000 000	4573 R	30 000 000
18					4573 L	21 000 000
20	2271 R	22 000 000	2304 R	280 000 000	4574 R	0
20					4574 L	500 000
22			2306 R	400 000 000	4575 R	184 000 000
22					4575 L	212 000 000

* Data of Hollander & Turner [11]

When the incubation periods in Table IV are plotted against the number of treponemes on a logarithmic scale (Fig 2) it is found that the groups of animals inoculated intradermally lie along a line with approximately a tenfold difference in the size of the inoculum corresponding to a 4-day difference in the length of the incubation period The groups of rabbits inoculated intratesticularly lie on a different line, but one which also shows approximately a tenfold difference in the inoculum corresponding to a 4-day difference in the length of the incubation period

In other words the data indicate that the treponemes in the testis and in the skin multiplied at about the same rate, but that a larger inoculum was required for the same incubation period in the testis by a factor of approximately 100 Some, but certainly not all, of this difference may be ascribed

FIG. 1 RATE OF MULTIPLICATION OF TREPONEMES IN RABBIT TESTES



Results of treponeme counts on testes of rabbits at various intervals after the inoculation of indicated numbers of T pallidum

Reproduced from Cumberland & Turner,[16] by kind permission of the editors of the American Journal of Syphilis, Gonorrhea and Venereal Diseases

10 ml of solution for 1 hour, after which the total number of treponemes present in the fluid was determined by darkfield counts of measured drops These data are detailed in Table III

That the response to inoculation is a function of the size of the inoculum is illustrated by the representative data of 64 animals whose incubation periods ranged from 5 to 42 days, depending on the size of the inoculum and the route of inoculation (see Table IV) The first section of Table IV lists the incubation periods of rabbits from two different experiments inoculated intradermally with 100-fold dilutions; the second section lists the incubation periods of rabbits inoculated intratesticularly with 250-fold dilutions; and the third section lists the incubation periods of rabbits inoculated with positive lymph nodes in an unselected series of infectivity tests These lymph-node emulsions were all darkfield negative and probably contained very few organisms The fourth section of the table shows the incubation periods observed in a consecutive series of intravenous inoculations

TABLE III NUMBER OF TREPONEMES EXTRACTED FROM NORMAL RABBIT TESTES AT VARIOUS INTERVALS AFTER INTRATESTICULAR INOCULATION OF 100 000 ORGANISMS *

Day	Experiment E		Experiment G		Experiment H	
	Rabbit testis	Number of treponemes	Rabbit testis	Number of treponemes	Rabbit testis	Number of treponemes
0	2766 R	0				
0	2766 L	0				
4	2773 R	0				
4	2773 R	0				
8	2767 R	2 000				
8	2767 L	2 000				
12	2772 R	500 000	2899 R	2 000 000	4570 R	1 700 000
12	2772 L	200 000			4570 L	2 400 000
14			2900 R	8 000 000	4571 R	5 000 000
14					4571 L	3 500 000
16	2768 R	7 000 000	2902 R	18 000 000	4572 R	11 750 000
16	2768 L	3 000 000			4572 L	3 750 000
18			2903 R	54 000 000	4573 R	30 000 000
18					4573 L	21 000 000
20	2271 R	22 000 000	2904 R	280 000 000	4574 R	0
20					4574 L	500 000
22			2906 R	400 000 000	4575 R	184 000 000
22					4575 L	212 000 000

* Data of Hollander & Turner [18]

When the incubation periods in Table IV are plotted against the number of treponemes on a logarithmic scale (Fig 2) it is found that the groups of animals inoculated intradermally lie along a line with approximately a tenfold difference in the size of the inoculum corresponding to a 4-day difference in the length of the incubation period The groups of rabbits inoculated intratesticularly lie on a different line, but one which also shows approximately a tenfold difference in the inoculum corresponding to a 4-day difference in the length of the incubation period

In other words the data indicate that the treponemes in the testis and in the skin multiplied at about the same rate, but that a larger inoculum was required for the same incubation period in the testis by a factor of approximately 100 Some, but certainly not all, of this difference may be ascribed

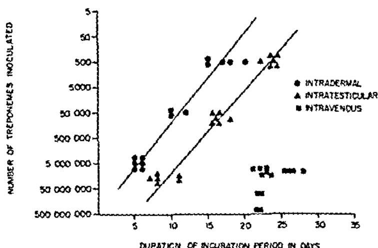
TABLE IV INCUBATION PERIOD IN DAYS ACCORDING TO THE SITE AND SIZE OF THE INOCULUM

Site	Intradermal		
Inoculum	500	50 000	5 000 000
Exp I [a]	18, 20	10, 10, 12	5, 5, 5
Exp II [a]	15, 17 17	10, 10, 10	6, 6, 6
Site	Intratesticular		
Inoculum	500	100 000	25 000 000
Exp III [a]	23, 25, 25 25, 25, 25	16, 16, 16 16, 16, 16	7, 8, 8 8, 11, 11
Site	Intratesticular		
Inoculum	*Lymph node emulsions*		
8 experiments [a]	23, 23, 23 26, 28, 28 28, 30, 30, 30 30, 30, 31, 36, 42, Neg		
Site	Intravenous		
Inoculum	10 000 000 to 750 000 000 (6 different)		
6 experiments [a]	21, 22, 22, 22 22, 23, 23 23, 23, 26, 26, 26, 28		

[a] Numbers refer to incubation period in days

to the greater ease with which minimal lesions can be seen and felt in the skin. More important may be a factor which can be designated " dilution ". It is deduced that much of the inoculum in the skin remains *in situ*, whereas much of the testicular inoculum spreads throughout the testis and also escapes in appreciable amounts from the testis via vascular and lymphatic channels It can be inferred, from the observation that an incipient orchitis is commonly a diffuse lesion of the testis, that inoculated treponemes spread freely through the testis It is interesting to speculate whether the presence of hyaluronidase in the testis can account for this difference in behavior between treponemes introduced intratesticularly and those injected intradermally

FIG. 2. RELATIONSHIP OF INCUBATION PERIOD TO SIZE AND SITE OF INOCULATION



Observed incubation periods after inoculation of measured numbers of treponemes by indicated routes in representative rabbits (Nichols strain), plotted from data in Table IV

Cumberland & Turner [14] observed a 93% reduction in the number of organisms recoverable from the testes during the first 2 hours, after the inoculation of 10 000 000 treponemes In order to show that some treponemes were actually escaping by either vascular or lymphatic channels

TABLE V DISPOSITION OF SPIROCHETES WITHIN FIRST FEW HOURS AFTER INOCULATION INTO TESTIS *

Spirochete counts	Testes removed and counts made immediately after inoculation		Testes removed immediately after inoculation and counts made 2 hours later [a]		Testes removed and counts made 2 hours after inoculation [b]	
	Normal	Immune	Normal	Immune	Normal	Immune
	11	3	3	4	16	7
Range	30 202	64 109	38-105	52 96	0 21	0-10
Mean	80	87	76	73	6	5
Median	80	89	86	71	6	5

* From Cumberland & Turner [14]
[a] One testis counted at 4 hours after inoculation
[b] Three testes counted at 4 hours, 2 testes at 8 hours, after inoculation

and not being merely destroyed *in situ*, the testes from one group were removed immediately after inoculation and spirochete counts were made at once In another group the testes were ligated at both ends immediately after inoculation, removed, allowed to stand in saline for 2-4 hours, and then counted In a third group of rabbits, the testes were inoculated, left undisturbed *in situ* for 2 hours, and then removed and counted

Table V (from Cumberland & Turner [16]) presents the counts obtained in the 3 groups of animals. It was apparent that the spirochetes were not destroyed in the resected testes in which the different vascular and lymphatic vessels were ligated, while in the intact unligated testes the counts dropped significantly during the first 2 hours.

The influence which dilution may have on the incubation period is also seen in the case of intravenous inoculation, whereby the inoculum spreads through the entire body. After treponemes are injected intravenously (Table IV), it is as though the inoculum were diluted to the degree that single treponemes, or at most clumps of treponemes, become the effective inocula, and multiple lesions are formed The incubation period then corresponds approximately to the expected theoretical period after inoculation of a single treponeme intradermally

With the same reasoning it is possible to explain why the incubation period after a lymph-node transfer is about 4 weeks. Lymph nodes contain relatively few organisms, and when these are diluted in the testis, even if a hundred or more are inoculated, the incubation period may approach that produced by inoculation of a single treponeme

Assuming that under optimum conditions the division time is 30-33 hours, and that 100 000 000 or more treponemes are required to form a perceptible lesion, it can be deduced, by extrapolation, that the longest time required for the development of a lesion under the conditions will be about a month. A single treponeme increasing tenfold every 4 days would reach this level in 32 days

After intradermal inoculation into the shaved backs of rabbits maintained in a cool environment the incubation periods approach the values given in Table IV. The incubation periods of 107 rabbits comprising 20 separate experiments, in which groups of 2 or more animals were inoculated intradermally with 500 organisms in multiple sites on the back, are listed in Table VI. The values show a remarkable uniformity, with the extreme figures grouped in single experiments, suggesting that errors in the methods of detection and enumeration of viable treponemes may be greater than variations in the response of animals The reproducibility of the incubation period is evidence in support of the opinion that the shaved backs of rabbits in a cool environment offer the optimum obtainable conditions for the growth of treponemes in rabbits.

Any incubation period much in excess of these values must indicate some interfering influence producing less than optimum conditions. We

TABLE VI INCUBATION PERIODS OF GROUPS OF RABBITS INOCULATED WITH 500 TREPONEMES INTRACUTANEOUSLY ON THE BACK

Number of group inoculated	Rabbit numbers	Number of rabbits	Observed incubation period in days	Range	Mean
1	22-08 13	6	18(4), 19(2)	18 19	18 3
2	23 76-81	5	16, 17(2), 19, 20	16 20	17 6
3	23-89 90	2	18, 20	18 20	19 0
4	24-85 90	5	16(3), 18(2)	16 18	17 2
5	25 19 21	3	17 19 21	17 21	19 0
6	26 29 30	3	17(2), 19	17 19	17 7
7	28 21-25	5	17, 18(4)	17 18	17.8
8	29 91 96	6	16(3) 17(3)	16 17	16 5
9	30 51-66	15	16(3) 17(10), 18(2)	16 18	17 0
10	29 35 39	5	17 18 19 20(2)	17 20	18 8
11	29-62-67	6	17 18(2) 19(3)	17 19	18 2
12	29-89 92	4	17 20 21 23	17 23	20 5
13	31 18-21	3	15 17 19	15 19	17 0
14	31 98 202	5	14(3) 15 17	14 17	14 8
15	33 58-61	4	20 21 22 24	20 24	21.3
16	32-48 54	7	13 14(6)	13 14	13 9
17	38-66 70	5	14 15(3) 16	14 16	15 0
18	38 71 79	9	13 14 15(2) 16(3) 17(2)	13 17	15 4
19	44 77-80	4	17(2), 18(2)	17 18	17 5
20	46-69-72	4	15(2), 16(2)	15 16	16 5

have observed an incubation period of 72 days in an animal (No 23-83) which was maintained in a warm environment, and in other animals receiving intermittent doses of penicillin [27] we have deliberately produced prolonged incubation periods of up to 167 days

Treponemal Infection of the Monkey

In the course of studies directed to assessing the potentiality of *T. cuniculi* infection as an immunizing agent against syphilis, limited observations were made on experimental treponematosis in monkeys Altogether 27 monkeys were inoculated 10 *Macaccus rhesus* and 4 African green *(Cerpithecus aetheopis sabaesis)* with the Nichols strain of *T. pallidum*, and 13 *M. rhesus* with *T. cuniculi*. Data were accumulated on the occurrence of

lesions, on organ infectivity, and on the serological reactions of blood and cerebrospinal fluid. Since the monkey did not prove to be a particularly suitable animal for the exploration of this problem, at least under the conditions of our laboratory, the experiments were not pushed to a conclusion Our observations will, however, be recorded for what they are worth

Comparison of Macaccus rhesus *and* Cerpithecus aetheopis sabaesis *(African green) monkeys*

Six monkeys of each species were inoculated with the Nichols strain of *T pallidum*, but 2 African green monkeys died early in the experiment. The inoculum consisted of an emulsion of infected rabbit's testis in which each 0.1 ml contained roughly 5 000 000 *T pallidum*. Each monkey was inoculated intracutaneously on the right thigh with 0.1 ml at one site, on the left eyebrow by scarification with 0 1 ml rubbed in with a cotton applicator, and in the preputial sac after scarification by 0.1 ml dropped into the sac and a cotton tampon inserted to aid retention of the inoculum. This same material was inoculated intracutaneously in 2 rabbits, both of which developed typical patterns of syphilomas after an incubation period of 8 days.

Evolution of lesions

By the 14th day after inoculation, 7 of the 10 monkeys had definite lesions at one site or another, and by the 17th day all animals had shown one or more lesions. The lesions were small infiltrated reddish or violaceous papules, in most of which *T. pallidum* could be demonstrated. The majority of the lesions developed within the first 3 weeks after inoculation, never reached more than 7-8 mm in diameter, and began to subside 2-3 weeks later, usually lesions developed at all 4 sites of inoculation on the thigh In general lesions developing on the eyebrow by scarification had a longer incubation period than those following intracutaneous inoculation.

On the 28th day after inoculation, when many of the previously observed lesions were beginning to subside, one monkey developed a small reddish papule on the mucous membrane surface of the prepuce. This was the only preputial lesion noted in the series.

Two months after inoculation all 6 of the rhesus monkeys and 2 of the 4 African green had shown darkfield-positive lesions. By the end of the 3rd month virtually all of these lesions had disappeared.

Careful search was made for the presence of generalized skin and mucous membrane lesions, in 2 animals (Nos. 21 and 23) an evanescent superficial scaly type of eruption was noted over the upper extremities and shoulders and head. The palms and soles were not involved. No treponemes were demonstrated in these lesions and their true nature remains obscure. A

summary of the pertinent clinical observations in the two small groups of monkeys is given in Table VII. Obviously the numbers of animals are too small to bring out anything but striking qualitative differences. The question in which we were interested was whether the African green monkeys are significantly more susceptible to syphilitic infection than *M. rhesus*; the data, while meager, suggest that they are not.

TABLE VII DATA ON CLINICAL COURSE AND SEROLOGICAL REACTION OF BLOOD (STS) AFTER INOCULATION OF MONKEYS WITH SYPHILIS TREPONEMES

Species	Monkey number	First lesions	Incubation in days	Titer of Eagle flocculation test on day indicated								
				0	29	67	97	124	164	194	210	361
M. rhesus	19	Eyebrow	21	0	0	2	4	1	4	4	4	8
	20	Thigh	17	0	-	0	4	8	4	8	8	4
	21	"	12	0	0	0	0	1	1	1	1	died
	22	Thigh and eyebrow	14	0	0	3	2	died				
	23	Eyebrow	14	±	0	0	±	4	2	12	2	1
	24	"	17	0	0	0	0	0	0	0	0	died
African Green	26	Eyebrow	12			2	8	8	2	4	4	2
	27	"	21	0	0	0	0	0	0	0	±	±
	28	Negative	—	0	0	0	0	0	0	0	died	
	29	"	—	0	0	±	0	0	0	0	died	

Blood and spinal fluid serology

Serological tests were made on the blood serum (Table VII) and cerebrospinal fluid of surviving monkeys. In approximately half of this small series of monkeys, blood serological tests became positive about 60 days after inoculation and remained positive for the following 8 months, always in low titer. In about half the animals the Eagle flocculation test remained negative, although some of the serum specimens from these animals showed a positive result when a more sensitive technique such as the Mazzini test was employed. Suffice it to say, however, that the rise in Wassermann antibody was at no time very marked in any of the inoculated animals, paralleling in a sense the rather minimal tissue reaction observed. The treponemal immobilization and agglutination tests were not available at the time of this study.

Examination of the cerebrospinal fluid obtained by cisternal puncture was made before inoculation and at various intervals after infection. The

examination consisted of cell count, Pandy test for protein, and a complement-fixation test for Wassermann antibody All tests were essentially negative and gave no indication of any syphilitic disease of the central nervous system Cell counts rarely exceeded 4 per ml and in only one instance was the count as high as 8 cells; these findings were considered to be within normal limits.

The infectivity of organs

Three of these animals died between the 5th and 8th months after inoculation, and the remaining 7 were sacrificed from 14 to 17 months after inoculation. Post-mortem examination revealed no gross changes suggestive of syphilitic infection The monkeys were sacrificed by exsanguination while under ether anesthesia. Suspensions of plasma, liver and lymph nodes were inoculated into the testes of 2 normal rabbits. In addition to the results of these inoculations shown in Table VIII, emulsions of heart muscle and cerebral cortex of monkey No 19, *concentrated by differential centrifugation*, were transferred to 2 rabbits, each with negative results, and unconcentrated emulsions of cervical and thoracic cord, cerebral cortex and medulla from monkey No. 28 were inoculated into 2 rabbits, each with negative results A combined unconcentrated emulsion of cerebral cortex and cervical cord of monkey No. 29 yielded positive results on transfer to rabbits

TABLE VIII ORGAN INFECTIVITY TESTS OF MONKEYS INOCULATED WITH SYPHILIS TREPONEMES RESULTS OF INOCULATIONS INTO PAIRS OF RABBITS

Monkey species	Monkey number	Results of infectivity tests on			
		Blood	Liver	Lymph nodes	Other
M rhesus	19	0, 0	+, +	0, died	Heart 0, 0 Brain 0, 0
	20	0, died	0, 0	0, 0	
	21	0, 0	+ 0, 0	0, +	
	22			+ +	
	23	+ 0	0 0	+, +	
	24	+, +	+, +	0, 0	
African Green	26	0 0	+ +	0, died	
	27	0, 0	0, 0	0, died	
	28	+, +		+, +	Brain 0, 0 Cord 0, 0
	29			+, 0	Brain-Cord +, +

Comparison of T. pallidum *and* T cuniculi *infection in* M. rhesus

Most of the monkeys inoculated with syphilis developed some more or less definite clinical evidence of disease, and in 10 of these *T pallidum* was demonstrated either by darkfield examination or by organ transfer In 9 monkeys standard serological tests became positive, while in 5 they were negative. Infectivity tests on the lymph nodes, which were often moderately enlarged, were positive in 7 of 14 monkeys, infectivity tests on the liver were positive in 4 of 7 tested, blood plasma in 3 of 8, and central nervous system tissue in 1 of 3 tested

Nearly all of the 13 animals inoculated with *T cuniculi* showed small lesions at one or more of the several sites of inoculation, but these lesions on the whole were quite insignificant, and in only 2 animals were treponemes demonstrated in the lesions Of 12 animals in which infectivity tests on lymph nodes were made, two were positive, one of these being an animal in which *T. cuniculi* had been demonstrated in a skin lesion. About half the inoculated animals developed low titer Wassermann antibody, while the other animals remained negative. There was no correlation between the results of these tests and those of the infectivity tests

Since the cuniculi lesions developing after intracutaneous inoculation of monkeys differ in appearance from syphilitic lesions in these animals, the following description of the cuniculi lesions is quoted from our record books under the date of 6 August, approximately 4 months after inoculation These lesions were noted first on 14 June, 2 months after inoculation

"Monkey 5—Right back—Lesions in Area 1 persist Definite pattern with largest lesions at sites 1 and 4 These appear as flat crusted lesions, the crust tending to be arranged in low spicules giving the impression of a group of tiny lesions When the crust is removed, a flat slightly raised pinkish lesion is seen This is largely covered by epithelium, with here and there a few tiny bleeding points Several darkfield preparations were made from the lesions at site 1, but no treponemes were seen after long search"

On the whole, lesions developing in monkeys after inoculation of *T cuniculi* tended to have a much longer incubation period and to persist for longer periods than those following inoculation of *T pallidum* For example, in the 5 monkeys lesions were noted as follows monkey No. 1—from the 96th to the 104th day after inoculation, monkey No. 2, 96th to 105th day, monkey No 4, questionable lesions only, monkey No 5, 60th to 104th day, monkey No 6, 56th to 90th day

No generalized skin or mucous-membrane lesions were noted in any animal, and there was no evidence of involvement of other organs

Yaws infection in monkeys

We have had only meager experience with yaws infection in monkeys and the reader is referred to the classical studies of Schöbl [58] for information

on this subject. The senior author attempted to establish strains of *T. pertenue* in a few monkeys by direct transfer of material from human beings with the disease [65] Of 5 monkeys inoculated thus, by the intracutaneous route and by scarification over the eyebrow, all showed slight but fairly definite lesions at the sites of inoculation, but *T. pertenue* was not demonstrated by repeated darkfield examinations or by occasional infectivity tests in rabbits.

Summary of studies on treponemal infection in monkeys

Several comments may be made concerning these experiences with treponemal infections in monkeys.

1. For the most part, inoculations of monkeys were made with strains of *T. pallidum* and *T. cuniculi* that were well adapted to rabbits It is quite possible that strains of these treponemes could be better adapted to primates, with the induction of more frank evidence of disease phenomena.

2 The monkeys on which the foregoing observations were made were being maintained in a distinctly unnatural environment, and were doubtless victims of intercurrent infections and other adverse circumstances to a greater extent than in the experiments conducted by Neisser [48] and Schöbl [58]

3. The results of these studies lead us to conclude that, apart from the costliness of the monkey in many countries, this animal is not well suited for the investigation of most questions pertaining to the treponematoses The difficulty of finding ready criteria of specific infection led us to abandon cross-immunity studies between syphilis and cuniculi infections in this animal species.

4. The fact that *T. cuniculi* can be shown to produce infection, both local and generalized, in the monkey, suggests that this species of treponeme may likewise be infective for human beings Only one reported attempt to infect man with this organism, with negative results, has come to the authors' attention.[37]

Treponemal Infection in Guinea-Pigs

Kolle & Evers [35] first demonstrated that guinea-pigs could be infected with *T. pallidum*, a finding later confirmed by Tani, Kakishita & Saito [63] Both of these groups of investigators noted occasional symptomatic infections, although in most animals the infection was entirely asymptomatic, and could be demonstrated only by transfer of lymph nodes to rabbits. Kato,[34, 45] however, observed lesions regularly in guinea-pigs and concluded that their susceptibility was intermediate between that of rabbits and that of rats and mice.

On the basis of the observation of 95 guinea-pigs inoculated with various species of treponemes, we have also come to the conclusion that this animal species is considerably more susceptible than the older literature would indicate. This experience will be briefly presented here

In the earliest series of guinea-pig inoculations carried out in 1940, of 32 animals inoculated by various routes with darkfield-positive material from rabbits infected with the Nichols strain of *T pallidum*, 12 developed darkfield-positive lesions and 3 others developed fairly typical lesions in which no treponemes were demonstrated In these pilot studies often inoculations were made in multiple sites, such as the skin of the back, prepuce and scrotum Different substances calculated to promote non-specific tissue reaction such as corn starch, sterile soil, and calcium chloride were included in the inoculum without clear-cut enhancing effect Mucin in 6% suspension was also employed

In a second series of guinea-pig inoculations made in 1950, of 13 animals inoculated intracutaneously both in the back and on the scrotum, all animals developed a well-defined lesion in one or the other site, 12 of 13 being positive on the back and 8 of 13 on the scrotum Of 6 guinea-pigs similarly inoculated with strain S6 all developed back lesions, and 3 of 6 scrotal lesions. Each of 2 animals developed back lesions upon inoculation with strain M.S.I.

Among 30 guinea-pigs inoculated intracutaneously on the back with yaws strain YD, 27 showed back lesions, while among 24 of the same animals which were inoculated also on the scrotum, 18 were positive, no animal failed to develop lesions in one or the other site The YD yaws strain was successfully maintained through 5 serial passages in guinea-pigs

Cuniculi A strain invoked back lesions in all of 8 animals, but not scrotal lesions

In order to determine whether or not the guinea-pig developed immunity in somewhat the same manner as the rabbit, 6 animals infected 6 months previously with the Nichols strain and 3 infected with strain YD were challenged with the homologous strain by intracutaneous inoculation No lesions occurred in any of the test animals, while normal controls developed lesions from the same inoculum

In all these experiments, the lesions developing in the guinea-pig were only slightly indurated and often showed only scaly surfaces, but were regularly darkfield positive In a few instances, however, typical indurated nodules developed following inoculation of syphilis spirochetes The regional lymph nodes have been found to be darkfield positive by other investigators [35] All in all, the guinea-pig appears to be somewhat less susceptible than the rabbit, and appears to offer no advantage as an experimental subject over the rabbit and hamster. It should be noted that no systematic study of the comparative reaction of the guinea-pig to various species of treponemes has been made.

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Treponemal Infection in Rats and Mice

Treponemes were first passed into rats and mice by Kolle & Schlossberger [38] in 1926. The extensive literature on mice experimentation has been reviewed by Gueft & Rosahn [24]

While our experience with these animals is limited, some of the earlier studies, as well as those of Rosahn, are relevant to our observations on the symptomless infections of hamsters For although symptomatic infections have been described in mice by Bessemans & De Potter [7] the characteristic infection in mice produces no gross changes Not only are lesions usually absent, but it is also difficult to demonstrate treponemes in the tissues, whether by tissue-staining or by darkfield, and *it is usually necessary* to resort to rabbit inoculation to demonstrate infectivity. This suggested to *Levaditi, Schoen & Sanchis Bayarri* [38] *the historically interesting theory* that there must be a filterable form in the life-cycle of the treponeme It was shown, however, principally by Kolle & Evers [35] for guinea-pigs, by Kato [32] for mice, and by Bessemans, Van Haelst & De Wilde [10] for mice and rabbits, that the infectious lymph nodes of the symptomless animals do in fact contain treponemes which can be demonstrated by darkfield. Gueft & Rosahn [24] in their discussion of this subject write that " success in finding the organisms appears to depend in large measure on the persistence of the observer."

Rosahn, Gueft & Rowe [51] reported that mice of all ages from birth to maturity were susceptible to treponemes After subcutaneous or intraperitoneal injection, the blood, skin, spleen, brain, or lymph nodes might become infectious for rabbits Lymph-node transfers to rabbits gave the highest ratio of recoveries and the shortest incubation periods. Brain tissue was least often infectious. No lesions were noted in the mice

Rosahn & Rowe [56] examined the infectivity of mouse lymph nodes for rabbits during the 2nd week and during the 12th and 13th week after the intraperitoneal inoculation of graded doses of treponemes The lymph nodes of mice examined after 2 weeks were infectious for rabbits where the original inoculum was 1 000 000 treponemes, or greater, but when examination was deferred until the 12th week, infectivity was demonstrable in mice which had received as few as 100 000 treponemes in the original inoculation.

Kato [33, 45] and Yasumoto [45 70, 71] in Matsumoto's laboratory made

inoculation They were regularly present from the 4th to the 8th week, and less often thereafter. Frequently only one or two organisms were seen in 250 microscopic fields. Yaws treponemes behaved in the same way.

Rosahn & Rowe[55] also developed an antibiotic assay method utilizing mice. This gave results comparable to those of the assay techniques of Turner, Cumberland and Li and of Rake, Dunham and Donovick (see Chapter 6). However, larger amounts of penicillin per gram body-weight were required for mice than for rabbits.

In a long-term study Rosahn[53] found that all of 5 mice were still infectious when examined 642 to 872 days after inoculation. While no lesions or tissue changes were found, there was a significant decrease in longevity in a large group of syphilitic mice, when compared with an untreated control group

Treponemal Infection in Hamsters

Bessemans and his group[5, 6, 8] reported that the golden hamster (*Mesocritus auratus*) developed symptomless infection with *T. pallidum* and *T. cuniculi*; that large numbers of treponemes could often be found in the regional lymph nodes; and that the treponemes could be passed from hamster to hamster Geiman & McKee[20] noted that hamsters inoculated with *T. pertenue* in addition developed extensive chronic skin lesions. Rosenau[57] in Geiman's laboratory has used the hamster as an experimental animal for the investigation of immunity and response to antibiotics. In our laboratory we have observed over 1100 hamsters inoculated with one or another species of treponeme

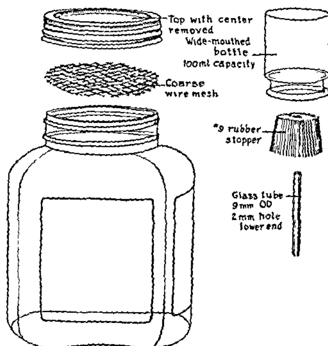
Technical considerations

The laboratory golden hamsters available in the United States are descendents of animals removed from a single Syrian burrow in 1930 The recent appearance on the market of color varieties indicates that in the future more variation may be expected in the species than occurred in the past Apart from a limited experience with " white " hamsters our animals have been the usual brown strain The white ones seem to have been equally susceptible to infection with treponemes, but are possibly less healthy than the standard strain, and more difficult to keep close shaved

Adult males were used almost exclusively, in conformity with the practice with rabbits. In general, the requirements for healthy animals apply to hamsters as well as to rabbits. In one respect hamsters, however, are preferable, since as far as is known they do not have a natural specific infection comparable to cuniculi infection of rabbits

The hamsters are housed individually in wide-mouthed jars, commonly used commercially for marketing sweet cakes or cookies. The screw-tops of these jars are modified by the insertion of a coarse wire mesh. Screw-tops are required to keep the animals from escaping. A small quantity of wood shavings is used for bedding. We customarily use commercially pr

FIG. 3 JAR AND DRINKING APPARATUS FOR MAINTENANCE OF HAMSTERS



food in the form of antibiotic-free mouse pellets which are placed loose in the bottom of the jar A continuous supply of water seems to be necessary for the health of the animals Water is supplied by an inverted bottle with a one-hole No 9 stopper and a short drinking tube (Fig. 3). The jars are cleaned once a week Hamsters remain good-tempered and gentle if carefully handled. A rubber-tipped forceps is used to lift them from the jar by gently grasping the loose skin of the neck. They are then set on a flat surface from which they can be picked up by grasping the same area with the fingers Hamsters which are roughly handled or which have painful lesions may become vicious

The same air-cooled animal-room was used for the rabbits and the hamsters. In experiments reported by Hollander & Turner [26] more lesions, more infections, and shorter incubation periods were observed in hamsters maintained at that temperature (18-21°C) than in identically inoculated hamsters maintained in a warm environment (29-31°C). (See Chapter 3)

Hamsters have a disadvantage in that they will occasionally begin to hibernate in the cool-room, and we have noted in agreement with Jahnel [29, 30] and Bessemans, De Wilde & De Moore [9] that treponemal infections regress during hibernation. Whether an infection may be cured by hibernation *per se* is not known.

Inoculations are usually made under ether anesthesia Satisfactory anesthesia can readily be induced by placing the hamster in a cookie jar containing a small amount of ether on some cotton Healthy animals are relatively tolerant of ether.

Intradermal inoculation has been used almost exclusively, either in the groin or on the back, or at both sites. Groin inoculations are usually made at a distance from the anus and genitalia to avoid involvement of these structures Inoculations about the face have been abandoned because of the frequent secondarily infected obstructive lesions of the nares Because of the individual variation in the sleeping habits of the hamsters, which may be found either curled up or sleeping on their backs, and because of the effects of local temperature on the development of lesions in the rabbit (Chapter 3), inoculations are often made in the groin and also on the back in order to provide two sites with perhaps slightly different temperatures. In most cases lesions have developed in both areas; in a few animals lesions have been limited to either the back or the groin, but neither site has been consistently superior.

One-ml tuberculin-type syringes and 3/8-inch or 1/2-inch No. 27 gauge needles are the most convenient for inoculations In order to obtain good intradermal blebs the needle is inserted through the full thickness of the skin and the dermis is re-entered from below The toughness of the outer layer of the skin makes it difficult to remain intradermal when puncturing directly from above

The usual diluent is 10% hamster serum in saline inactivated at 56°C for 30 minutes and diluted with 15% glycerol

When active skin lesions are present, large numbers of treponemes are found at their margins, however, since emulsions of these areas are often contaminated, and in the case of open ulcerated lesions, heavily contaminated, the lymph node is a preferable tissue for transfer Lymph-node transfers have been consistently successful with all strains of syphilis, bejel and yaws, except the Samoa yaws strains, which rarely show treponemes in the lymph nodes. Samoa strains have had to be maintained in passage by transfer of material from the skin lesions

Clinical course of infection in hamsters

The manifestations of disease in hamsters appear to be confined to the skin and lymph nodes No internal lesions have been identified, even in animals with very extensive superficial lesions Hamsters with extensive lesions may become very sick, and some have died presumably because of the infection, although in the latter cases extensive and obviously painful lesions, or lesions involving the mouth and nares or the anus, were present, and the immediate cause of death was believed to have been lack of food and water

TABLE IX INCUBATION PERIOD AFTER INTRADERMAL INOCULATION OF HAMSTERS WITH INOCULA OF VARIOUS SIZES (HAITI B STRAIN OF YAWS)

Number of treponemes in inoculum	Experiment I		Experiment II	
	Hamster number	Incubation in days	Hamster number	Incubation in days
250 000	799 800	17 15	815 816	14 16
25 000	801 802	19 21	817 818	21 20
2 500	803 804	27 27	819 820	26 21
250	805 806	30 28	821 822	33 26

Skin lesions at the site of inoculation appear after an incubation period which is related to the size of the inoculum. This is illustrated by two series of hamsters inoculated with tenfold dilutions of emulsions of hamster-adapted Haiti B strain of treponemes It is observed in Table IX that there is an increase of approximately four days in the incubation period for each tenfold dilution of the inoculum Since this is the same amount of prolongation produced in rabbits by a similar decrease in inoculum, it may be inferred that the treponemes multiply in hamsters at approximately the same rate as in rabbits. The incubation periods, however, are much longer in hamsters when inocula of the same size are compared, suggesting either that the number of treponemes required to produce lesions in hamsters is greater than it is in rabbits, or that there is a prolonged time-lag before multiplication begins. Of an unselected group of 191 hamsters which developed lesions after the inoculation of one of 11 strains of yaws or bejel or endemic syphilis, 27% had incubation periods of 30 days or over. In 4 hamsters of this latter group, the incubation periods lasted for more than 60 days.

Involvement of lymph nodes

Much of our knowledge of experimental treponemal disease of hamsters has been made possible through the realization that the lymph nodes play an important role in the infection. The inguinal nodes have been found to be excellent sources of treponemes after groin inoculation of any of our recently acquired strains of syphilis, endemic syphilis, bejel and all strains of yaws except those from Samoa.

While normal hamster nodes are small and insignificant, the infected nodes become prominent and are easily dissected from the inguinal fat just lateral to the external femoral vein. For convenience, the animals are usually sacrificed and the nodes removed post mortem, however, it is also practicable to remove one or both nodes under ether anesthesia. As an illustration, two separate operations performed on a hamster inoculated with a strain of endemic syphilis from Bosnia had no noticeable effect on the course of the disease, and treponemes were still demonstrable in the axillary nodes one year after the original infection.

The number of treponemes in the inguinal nodes reaches a peak about 4-6 weeks after inoculation, or even later with small inocula Thereafter the number of treponemes slowly decreases until they may be difficult to find after 3 or 4 months Positive darkfields, however, have been obtained after a year, and infectivity of the nodes has been demonstrated consistently after the same period The other superficial lymph nodes of the body begin to become positive at about the time that regional nodes are most involved, although the former nodes do not ordinarly develop large numbers of treponemes or show much enlargement

TABLE X APPROXIMATE NUMBER OF TREPONEMES PRESENT IN THE INGUINAL NODES OF HAMSTERS AT INTERVALS AFTER THE INTRADERMAL INOCULATION OF 500 000 TREPONEMES IN EACH GROIN *

Week after inoculation	Strain of treponemes		
	Syphilis Chicago	Yaws Haiti B	Bejel Syria B
1	2 000	2 500	0
2	950 000	76 000	25 000
3	4 250 000	4 775 000	272 000
4	10 262 000	6 175 000	2 771 000
5	191 500	1 025 000	30 187 500
6	45 000	147 000	6 662 500
8	431 000	4 350 000	1 250 000

* Each figure is the arithmetic average of the results in two animals

Table X shows the extraordinary number of treponemes which may be present in the lymph nodes at the peak of the infection With each of the 3 strains mentioned, 16 animals were inoculated with approximately 500 000 treponemes in each groin At the designated intervals 2 animals were sacrificed, the inguinal lymph nodes were emulsified in a small measured quantity of diluent, and the total number of treponemes contained in the lymph were approximated by the usual darkfield method of enumeration

Comparison of different strains of treponemes in hamsters

A feature of treponemal infection in hamsters is the variation in the occurrence of skin lesions after intradermal inoculation. The strains which we examined fall into 3 general categories on the basis of the relative propensity for involvement of the skin and lymph nodes. The syphilis strains regularly involve the lymph nodes, but produce, as a rule, little or no reaction in the skin. This picture has been designated the Sh type. The Samoa strains of yaws, on the other hand, produce severe, expanding skin-ulcers but the lymph nodes ordinarily do not contain treponemes. This picture has been designated the Yh type. Most of the strains of yaws, bejel, and endemic syphilis are intermediate They frequently produce chronic skin lesions, and regularly show involvement of the lymph nodes This picture in hamsters has been characterized as the Mh type

The nature of these differences can be examined in the 533 animals listed in Table XLVII (see page 199) Most of these hamsters have been inoculated since 1953, from which date the procedure of inoculation and examination has remained essentially the same.

Syphilis type of reaction in hamsters (type Sh) Six strains, including YD-post-1949, characteristically produced symptomless infections in hamsters No lesions were seen in 128 of the 153 animals in this group Of 6 animals which developed indurated crusted ulcers very similar to typical syphilitic chancres in rabbits, 2 had been inoculated with Nichols strain, 2 with Chicago and 1 each with Baghdad A and Baghdad B strains. In 11 animals transient scaly insignificant papules or small crusts appeared after about a month and persisted for periods which ranged from a few days to a little over a week. It is believed that more lesions of this type might have been discovered with more detailed or more frequent examinations

These lesions seemed to be more frequent with Baghdad A than with the other strains, and were present in animals subjected to the 7th, 9th, and 10th passages of this strain With the other strains these lesions did not appear more frequently after multiple passages Twelve animals developed papules which seemed to begin at the time of inoculation. These usually regressed within 2 weeks, but 2 animals had persistent papules for 24 days (H-886) and 45 days (H-497), respectively A possible relationship of these papular early lesions to foreign matter in the inoculum is suggested by their development in 2 animals (H-885 and H-886) that were inoculated with an emulsion to which particles of soluble starch had been added, and their absence in 2 controls receiving the unadulterated inoculum While the appearance of such lesions may have been related to some irritant in the inoculum, this does not mean that they were not specific lesions, and actually treponemes were readily demonstrated.

Despite the absence of skin lesions, the inguinal nodes in this group were consistently enlarged, infectious, and almost always darkfield positive.

Treponemes were found in the lymph-node emulsions of 130 of 144 animals examined. Six of the negative nodes, however, produced infections when inoculated into normal hamsters, and it seems reasonable to believe that all the nodes actually contained treponemes Four of the negative examinations were after 2 months, 8 after 3 months, and 2 after 4 months

Yaws-bejel type of reaction in hamsters (type Mh) Three strains of yaws, 2 of bejel and 4 of endemic syphilis from Bosnia and Bechuanaland, when inoculated into 211 hamsters, presented a rather different picture from that described above Each strain produced skin lesions in the majority of the animals, and with most of the strains these lesions appeared with great regularity In addition, many of the animals which were observed for longer than 6 weeks entered a chronic stage in which the periphery of the lesion progressed while the center healed The lymph-node involvement of these animals was quite like that of the syphilis group except that the nodes were often larger No individual differences among these strains have been recognized

The typical course in hamsters is as follows After a variable incubation period, a zone of slightly indurated erythema, 0 5-1 0 mm across, appears at the site of the inoculation This progresses quickly within a week to a stage of roughening and scaliness, and then to ulceration or to crusting. The central area remains unchanged for a week or two while the periphery expands, the lesion becoming either a larger open ulcer or a larger crusted lesion The crusts or open ulcers may continue to expand, but more often there is central healing and the only sign of activity is at the margin, which then has a distinct annular configuration After about six months part of this may be healed with only a few disconnected linear or serpiginous areas of activity not clearly connected with the original site These become less and less noticeable and eventually disappear Throughout the course of the infection treponemes can be demonstrated in the active margin of the expanding lesion An occasional animal develops metastatic lesions about the mouth and nares, and one darkfield-positive ulcer of the tongue was seen in a Syria A hamster

In this yaws-bejel group many more animals would probably have developed extensions had they been kept under observation for longer periods Doubtless more metastatic lesions would also have occurred The prevalence of darkfield-negative lymph nodes was low, as in the syphilis group, in this group also, some of the darkfield-negative nodes were transferred to other hamsters and found to be infectious

Samoa yaws type of reaction in hamsters (type Yh) The 3 Samoa yaws strains studied in 13 to 16 passages in 129 animals induced a disease pattern in hamsters unlike that brought about by any of the other yaws strains, in that the lymph nodes were almost always darkfield negative Of 88 hamsters in which the lymph nodes were examined only 6 were darkfield positive,

in 3 of these 6 only a single treponeme was found, and in the other 3 only 2 treponemes. Moreover, only 6 of 18 lymph-node transfers from hamsters infected with Samoa strains were successful, one of these being a darkfield-positive node

By contrast, the transfer of emulsions of the lesions is consistently successful and the strains can be passed serially without difficulty. Although the lesions are usually grossly contaminated open ulcers, the inoculum does not seem to produce a purulent reaction, and the new lesions start as typical indurated or scaly lesions The Samoa treponeme strains, in fact, seem to be more virulent for the hamster than the other strains studied Almost all progress to coalescent open ulcers, and 12 cases which were observed for as long as 6 months showed extensive metastases consisting of scaly indurated and crusted lesions involving the soles of the feet, the tail and the nose. Because of the longer period during which some of these Samoa animals were observed it cannot be stated with certainty that similar lesions would not have been found with the same frequency in some of the other strains, had the latter been observed for equal periods.

Cuniculi infection reaction in hamsters Altogether 25 hamsters have been inoculated with emulsions of strain cuniculi A Positive lymph nodes have been found in 11 of 23 examined, and one animal has been observed with crusted skin lesions Attempts to adapt this strain to hamsters and to learn whether its behavior in hamsters is distinguishable from the other type have not been altogether successful It is suspected that the cuniculi strain may be less virulent for the hamster than the other strains of treponemes studied However, since studies with this strain were started later, no definite conclusions can be drawn at this time.

Treponemal Infections in Other Animals

There are a number of claims in the literature of successful inoculation of various species of animals other than human beings, monkeys, rabbits and the small laboratory animals. While, for most of these, rigorous proof of susceptibility is lacking, the total evidence suggests that many animals are susceptible to treponemes Indeed it is possible that the development of infections may depend not on the animal species, but wholly on suitable local conditions of temperature, oxygen levels, and similar factors, which are discussed later Dogs, cats, sheep, and heifers are among the animals which it is claimed have been infected, in each case by investigators familiar with the appearance of the disease in other animals. Hoffman & Bruning[25] inoculated material from an early human syphilitic chancre into the anterior chamber of a poodle dog A keratitis developed after 16 days and the iris later became involved; 26 days after inoculation the lesion was healing. A second inoculation was made in the eye of a Spitz

dog from a human primary lesion This dog developed a keratitis after 21 days. Typical spiral organisms were demonstrated in Giemsa-stained smears of the lesion.

Bertarelli,[4] who was the first to demonstrate the infectivity of treponemes for rabbits, inoculated material from the scrotal lesion of a rabbit into the anterior chamber of a sheep, and into the scrotum and the anterior chamber of a dog; 16 days after inoculation both animals developed lesions which were interpreted as syphilomas. Material from the eyes of these animals produced typical lesions when transferred back into rabbits Bertarelli also observed a suspicious skin lesion in a pig, 3 weeks after inoculation, but did not further pursue this line of investigation

Levaditi & Yamanouchi[39] succeeded in transmitting treponemes to cats in 2 experiments A strain furnished by Bertarelli which had been passed many times in the rabbit was introduced by infected rabbit corneal tissue into each of 3 suckling cats After a few days the acute inflammation subsided. One animal sacrificed at 18 days was essentially negative After 40 days the second animal began to show a specific keratitis, consisting of an opaque vascularized area 4 mm across A biopsy on the 46th day, when stained with silver, showed a dense infiltration of treponemes The third animal showed a lesion persisting at the time of the report

Béclère[3] reported experience with the inoculation of treponemes into 5 young calves In each of the 5 animals the inoculation of rabbit-adapted syphilis treponemes of the Truffi strain was followed by the development of little papules after incubation periods of 3-4 weeks In 3 animals inoculation of human material and in 1 animal inoculation of passage material from a heifer gave the same kind of reaction Treponemes were demonstrated microscopically in 4 of the 6 animals, but infection was not proved in the other 2 animals The best lesions developed in a 5-day-old heifer

The Susceptibility of Mammalian Species to Induced or Natural Treponemal Infections

Although the rabbit and hamster, and to a less extent the monkey, have been the host species commonly employed in experimental treponematosis, it is apparent that there are many other animal species susceptible to treponemal organisms Indeed, it seems fair to say that no mammalian species has been found wholly and naturally resistant to some of the treponemes that are considered to be primarily human pathogens

To what extent some naturally occurring treponemal disease similar to venereal spirochetosis of rabbits may exist among other mammalian species can only be a subject of speculation No such syndrome has been reported, but on the other hand no systematic search for such infections seems to have been made either among common domestic animals or among

wild species The newly developed specific serological tests such as the treponemal immobilization and the treponemal agglutination tests may provide a method for gaining information bearing on this problem

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infection with the Nichols strain of *T pallidum* than were English, Himalayan or Rex breeds The main differences found were in the course of the infection and in the incidence and distribution of generalized lesions The mean incubation period for the primary orchitis was fairly constant, with minor variations which were probably not significant

Most of our studies on rabbits have been made with mixed breeds, mainly albinos of unknown ancestry We have suspected that healthy rabbits of all breeds are uniformly and almost equally susceptible At one point in our studies it became convenient to use a highly inbred strain of Dutch Belt rabbits, and, despite the studies referred to above, these seemed to be about as susceptible as other breeds in use at that time in the laboratory.

Age and sex

Chesney [11] found that a small group of young males inoculated intratesticularly showed a slightly more marked initial lesion and a tendency to postponement of subsequent generalized lesions, when compared with a group of adult males similarly inoculated In this laboratory adult rabbits have been used almost exclusively

Male rabbits have been used partly because of the convenience of the intratesticular route of inoculation, and partly as a result of observations such as those of Chesney [11] who found that the response to intradermal inoculation was distinctly less marked in females than in males, and the extensive observations of Magnuson, Rosenau & Greenberg [27] who deduced that, in the male, either the division time of the organism is shortened, or the local reaction is heightened To what extent this difference is attributable to hormonal influence is not clear These and other studies on the relationship of sex hormones to the course of experimental syphilis, including reports by Kemp & Shaw,[30] Kemp, Shaw & Fitzgerald [31] and Frazier, Mu & Hu,[23] have a new significance, perhaps, in the light of the demonstrable effect of cortisone and ACTH on treponemal infection Again the possibility that observed differences may be related to differences in body and skin temperatures of males and females must be considered

Health

It is generally agreed that experimental treponemal infections are more readily induced in healthy than in unhealthy animals, in the latter, lesions may develop poorly or not at all Much of this different behavior may be ascribed to elevation of the body temperatures of the sick animals

The interfering conditions of an infectious nature that are most frequently encountered are those manifesting themselves as diarrhea, caseous abscesses or ear canker

Chapter 3

FACTORS AFFECTING THE EVOLUTION OF EXPERIMENTAL TREPONEMATOSIS

In the experimental animal as well as in the human being the course of treponemal infection can be regarded as an interaction between the infecting treponeme on the one hand and the host on the other. Since, however, both parasite and host are subject to modifying influences the component parts of this interaction are often obscured It should be the aim of the investigator to segregate one or another of these influential factors in order to evaluate its effect upon the over-all picture of the disease.

Most studies of this sort have been made with the rabbit as the host species and *T pallidum* as the infecting organism, and our discussion will be largely limited to this system, but, in general, it may be anticipated that the findings will be applicable to other susceptible animals and to other pathogenic treponemes

The Rabbit Host

Breed of rabbit

In the light of recent work on the effect of the genetic constitution of the host on the response to infectious agents, it would not be surprising if differences could be demonstrated in the response of inbred lines of rabbits to treponemal infection. Actually, however, very few such observations have been made; moreover, the differences which have been described may stem from secondary effects rather than from a fundamental alteration in the host's susceptibility. For example, as will be pointed out later, the mul- [illegible] of the [illegible] n color [illegible] racters may thereby give rise to alterations in the internal environment which are sufficient to modify the evolution of the disease.

With the foregoing limitations in mind it should be noted that Rosahn [51] reported that Havana and Dutch breeds of rabbits were more resistant to

infection with the Nichols strain of *T pallidum* than were English, Himalayan or Rex breeds. The main differences found were in the course of the infection and in the incidence and distribution of generalized lesions. The mean incubation period for the primary orchitis was fairly constant, with minor variations which were probably not significant

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The interfering conditions of an infectious nature that are most frequently encountered are those manifesting themselves as diarrhea, caseous abscesses or ear canker.

Organisms of the *Pasteurella* group are endemic in many rabbit colonies, frequently causing acute or chronic infections, which may reach epidemic proportions. Diarrhea, elevated body temperature, and loss of weight are the principal manifestations of the acute disease, while single or multiple caseous abscesses are characteristic of the more chronic form.

Ear canker or scabies, characterized by scabs and superficial ulceration of the inner surface of the external ear, is caused by an infestation with a small mite. In its early stages this condition readily responds to the application of a few drops of mineral oil or kerosene, the latter, however, being more irritating. If neglected, this condition is difficult to treat and may lead to disease of the internal ear, and to involvement of the vestibular apparatus causing disturbances of equilibrium, finally leading to meningitis and death.

Food and housing

Rabbits in this laboratory have been maintained on commercial compressed rabbit rations and water as desired. These rations, in general, furnish an adequate diet, but seem to be deficient in salt. When the rabbits are given compressed salt " spools " in addition to their rations they appear healthier, eat better, gain weight faster, and have less diarrhea.

The presence of antibiotics in the rations is a serious matter in the experimental treponematosis laboratory. It is now difficult to procure prepared animal food in North America which does not contain one or another antibiotic, either purposefully added or inadvertently included from some remote source. As will be pointed out in Chapter 6, pathogenic treponemes are sensitive in greater or less degree to most of the antibiotics now on the market, and many of our experiments have been vitiated owing to the presence of antibiotics in the animal food, at one period undetected antibiotics led to repeated failure in source treponemes for the TPI test.

Few direct experiments have been made on the effect of diets on experimental treponematosis. Otsuji,[39, 42-44] however, reported that diets inducing acidosis have an enhancing effect on the development of experimental syphilis after intravenous inoculation in the rabbit, while diets inducing alkalosis lead to a prolongation of the incubation period and somewhat fewer and smaller lesions. Otsuji,[39, 45, 46] also reported that the inclusion of lanolin in the rations has an inhibitory effect on the development of syphilitic lesions in the rabbit. We have not attempted to repeat these observations

Our animals are customarily housed in individual cages made of metal wire. The use of separate cages prevents fighting, which can lead to confusing traumatic lesions. We prefer solid-bottom cages, with bedding of straw, wood shavings or sawdust, to cage-bottoms made of perforated metal or wire, which are easier to keep clean but tend to cause confusing

abrasions and ulcerations on scrota and feet, particularly in animals maintained for more than a few weeks.

The temperature of our animal-rooms is routinely kept at 20°C (68°F), or slightly lower, by an air-conditioning unit. The necessity for this procedure will be brought out in the next section Variation of the temperature between 18°C and 21°C is permitted, although a constant temperature would undoubtedly be more desirable.

Influence of Temperature

Temperature seems to be one of the most influential of the environmental factors which affect the course of treponemal infection, the development of lesions being related very critically to the temperature which is optimal for the growth and multiplication of treponemes. Attention was first directed to temperature by the observation that the severity of experimental infections fluctuated with the seasons of the year

Effect of environmental temperature

Almost everyone who works with experimental syphilis is impressed with the fact that lesions are more frequent and more severe in winter Brown & Pearce [8, 9] believed that the resistance of rabbits is altered by an environmental factor, probably sunlight Matsumoto [38] suggested that some "internal metabolic changes" occur

Yamamoto,[58, 61] of Matsumoto's laboratory, attempted to reproduce the seasonal changes by artificial control of the environmental temperature Animals maintained at natural summer or winter temperatures were compared with others inoculated at the same time but maintained at contrasting temperatures In the summer, 1 animal remained asymptomatic and a second showed small transient lesions, whereas 4 animals maintained in a cool environment developed rapidly progressive lesions In the winter, 3 animals developed rapidly progressive lesions, while 4 of 6 maintained at 28°C remained asymptomatic and showed transient lesions

The general manner in which the environmental temperature influences the course of experimental syphilis was reaffirmed in experiments reported by Hollander & Turner [28] (see Table XI) When 19 rabbits were inoculated with 500 treponemes at multiple sites on the shaved surfaces of the back, and were maintained in a cool environment (18-21°C), each developed a complete pattern of 6 lesions within 20 days. On the other hand, 18 rabbits with corresponding inoculations maintained in a warm environment (29-31°C) developed lesions poorly or not at all Six of the 18 in the warm-room remained asymptomatic, and only 12 developed lesions after long incubation periods of 21 to 72 days, half of these showing less than the full pattern of lesions

TABLE XI INFLUENCE OF ENVIRONMENTAL TEMPERATURE ON THE INCUBATION PERIOD OF RABBITS INOCULATED WITH "T PALLIDUM" AT SIX SITES ON THE CLIPPED BACK (INOCULUM, 500 TREPONEMES PER SITE) *

	Cool-room (18°-21°C)			Natural summer temperature (27°-35°C)		
	Rabbit number	Incubation (days)	Number of lesions	Rabbit number	Incubation (days)	Number of lesions
Experiment I	23-80	16	6	23-86	21	6
	23-79	17	6	23-87	25	5
	23-81	17	6	23-82	27	2
	23-77	19	6	23-83	72	2
	23 76	20	6	23-85	X	0
Experiment II	24-85	16	6	24-80	23	6
	24-86	16	6	24-81	23	5
	24-88	16	6	24-83	23	6
	24-87	18	6	24-84	25	6
	24-89	18	6	24-82	44	2
	24-90	18	6	24-79	Died	

	Natural winter temperature (18°-21°C)			Warm-room (29°-31°C)		
	Rabbit number	Incubation (days)	Number of lesions	Rabbit number	Incubation (days)	Number of lesions
Experiment III	44 77	17	6	44 75	22	6
	44 78	17	6	44 76	24	6
	44 79	18	6	44 73	X	0
	44-80	18	6	44-74	X	0
Experiment IV	46 70	15	6	46-60	28	4
	46 72	15	6	46 57	X	0
	46 69	16	6	46 58	X	0
	46-71	16	6	46 59	X	0
Experiment IVA	46 61 [a]	19	6	46-68 [b]	16	6
	46-63 [a]	20	6	46-66 [b]	17	6
	46-64 [a]	20	6	46-67 [b]	17	6
	46 62 [a]	21	6	46-65 [b]	18	6

* From Hollander & Turner [36]

X = popliteal lymph nodes not infective when passed to other rabbits after 90 days

a These animals were maintained in the cool-room for the first 8 days after inoculation and were then transferred to the warm-room

b Animals in warm-room for 8 days and then transferred to cool room

In one experiment some rabbits were kept in the warm-room for only a portion of the incubation period. These animals had a response which was intermediate between those kept for the whole period either in the warm-room or in the cool-room. An intermediate response resulted whether the stay in the warm-room was immediately after inoculation or later in the incubation period.

Hamsters inoculated with various strains of syphilis, bejel, or yaws, likewise responded better in the cool-room than in the warm. It will be noted in Table XII that, in those animals developing lesions, the incubation period tended to be longer among those in the warm-room, and that a larger proportion of the warm-room animals showed no evidence of infection. Infected lymph nodes of the cool-room animals were consistently larger and contained more treponemes than those of the warm-room hamsters. In the experiments for which data are given in Table XII the two groups of hamsters for each strain of treponemes were inoculated simultaneously with the same material.

TABLE XII INFLUENCE OF ENVIRONMENTAL TEMPERATURE ON THE COURSE OF TREPONEMAL INFECTION IN HAMSTERS *

	Strain	Species	Cool room (18°-21°C) [a]				Summer temperature [a] (29°-31°C)			
1	Baghdad A	Syphilis	+	+	+	+	+	+	+	+
2	Bosnia A	Endemic syphilis	11	11	17	19	26	28	40	42
3	Bosnia B	Endemic syphilis	10	20	32	38	25	28	+	+
4	Chicago	Syphilis	23	+	+	D	+	+	+	+
5	Haiti A	Yaws	+	+	+	+	+	0	0	0
6	Haiti B	Yaws	8	9	D	D	+	+	+	D
7	Indonesia B	Yaws	13	13	25	30	32	43	52	0
8	Iraq B	Bejel	41	47	+	+	0	0	0	0
9	Nichols	Syphilis	13	15	25	+	+	+	+	+
10	Syria A	Bejel	+	+	+	+	+	+	0	0
11	Syria B	Bejel	25	39	+	+	+	0	0	0
12	YD-post 1949	Yaws	+	+	+	0	+	+	+	+

* From Hollander & Turner *
+ = *positive inguinal lymph nodes — no local skin lesion*
O = negative inguinal lymph nodes — no local skin lesion
D = died
[a] Numbers denote incubation period of local skin lesions in days

The seasonal temperature influences the length of the incubation period of treponemal infection in rabbits and hamsters. The shortest incubation periods are observed in winter, or when the environment is cool. Artificial

elevation of the environmental temperature in winter reproduces a situation and a disease like that ordinarily seen in summer, and, conversely, artificial cooling in the summer can reproduce the winter type of disease. A temperature of winter rather than of summer character is required for the optimum production of treponemal lesions in rabbits and in hamsters.

Effect of local tissue temperature

The upper temperature level at which treponemes are able to survive in tissue has been established in experiments directed toward the problem of fever therapy in human syphilis Weichbrodt & Jahnel[59] in 1919 found that testicular syphilomas healed quickly when infected rabbits were placed at 41°C for periods of half an hour Schamberg & Rule[52, 53] observed in a few animals that daily hot baths were able to suppress the development of lesions, or cause rapid healing, but that the lymph nodes might remain infectious.

Bessemans & Haequaert[2, 4] found that lesions fail to develop in the testis, a normally susceptible tissue, if after inoculation the testis is pushed up into the warmer abdominal cavity for a few hours Turner (unpublished observations) noted that only 2 of 11 rabbits inoculated intraperitoneally with the Nichols strain of *T pallidum* were infected when tested by lymph-node transfer.

Hollander & Turner[29] reported observations on the number of treponemes in the testes of rabbits during the incubation period when the local temperature was altered. The progress of the infection was followed by removal and extraction of individual testes at intervals (see Table III, page 45)

It is interesting that when this technique was employed little difference was noted in the rate of increase in animals maintained in the warm- and cool-rooms It has been observed that in general there is no marked difference in the incubation period of testicular lesions of animals in warm- and cool-rooms, although there is a significant variation in the size and evolution of the testicular syphilomas

The difference in behavior of syphilitic lesions following intradermal and intratesticular inoculation may be due in part to the temperature-regulating mechanism of the testis described by Moore & Quick.[40] These investigators have shown that the temperature of the mammalian testis is normally maintained at a level substantially below that of the abdominal cavity, regulation of testicular temperature seems to be controlled by the muscles of the scrotum which elevate or lower the testis. In warm environments, therefore, the temperature of the testis may be relatively lower than that of the skin.

Attempts were made by us to elevate the testicular temperature by placing the testes within the abdomen No treponemes were found after

successful retention of the testes by sutures for a few days or after section of the gubernaculum and mesenteric attachment.

Since these intra-abdominal testes were, however, distinctly atrophic, the results may not have been due entirely to the temperature differences Moore & Quick [40] found that tubular epithelial cells of the testis degenerate at the temperature within the abdomen, and it is of interest that Frazier, Hu & Ma [20] reported that rabbit testes which are atrophic as a result of the injection of estrogenic hormones do not develop orchitis on direct inoculation with syphilis treponemes.

Bessemans [2, 3] and his group concluded that treponemes are "thermophobic" and "frigitropic"—that is to say, they have little resistance to high temperature but rather prefer cool tissues Bessemans noted that when the tissues of the rabbit are arranged according to temperature (Table XIII), those with the lowest temperatures include areas in which lesions are most likely to develop, and the areas with temperatures higher than that of the inguinal and popliteal nodes do not usually contain treponemes Bessemans & Van Canneyt [5] noted a similar correlation between the temperatures and the susceptibility of various areas of the eye.

Experiments reported by Hollander & Turner [29] in which inoculations were made in the ears of rabbits maintained at different temperatures suggest that it is not simply a low temperature which is required but an optimum temperature range, apparently of the order of 30-35°C. Ear-temperature differences were produced by unilateral cervical sympathectomy, which, as Claude Bernard [1] demonstrated in 1851, causes vasodilatation of the ipsolateral side of the face and ear A significant difference in ear temperatures (Table XIV) was obtained when the sympathectomized animals were maintained in the cool-room When these rabbits were inoculated intradermally on both ears, lesions developed in the warm rather than in the cold ears

It can be inferred that the optimum temperature range for growth of treponemes is rather limited, and is higher than the temperature of normal rabbit ears in a cold environment, but is less than the internal body temperature of the rabbit

The rather restricted optimum temperature range of the treponeme suggested by these experiments, coupled with the temperature gradients of the body (Table XIII), may well account for the variation of susceptibility of different tissues to treponemes, whether at the site of inoculation or elsewhere

The distribution of generalized lesions, which are frequently encountered after local testes or skin inoculations, has been described in detail by Brown & Pearce [7] by Hashiguchi,[26 28, 38] and by Bessemans [2] They occur principally in the testis, in the skin, most often of the feet, legs, head, face and tail; at the mucocutaneous borders of the nares, lips, eyelids, genitalia, and anus, in the bones, most commonly of the face, feet and legs, and in

TABLE XIII LOCAL TEMPERATURE MEASUREMENTS IN THE RABBIT ARRANGED IN ORDER OF INCREASING TEMPERATURE *

Site	Temperature (°C)
Laboratory	21.65
Skin of paw pad	33.37
Cornea, eyes open	34.55
Nasal bone	34.75
Outer surface of ear	35.17
Lower eyelid	36.23
Frontal bone	36.42
Base of ear	36.85
Surface of testis	37.34
Parietal bone	37.35
Brain	37.61
Skin of abdomen	37.78
Popliteal nodes	38.05
Abdominal wall	38.23
Axillary nodes	38.25
Inguinal nodes	38.28
Intra-abdominal testis	38.44
Lungs	38.45
Lumbar muscles	38.52
Mouth	38.52
Intercostal muscles	38.73
Liver	38.86
Abdominal cavity	38.95
Rectum	38.98

* Adapted from Bessemans [3]

the eyes. It will be noted from Table XIII that in these areas the local temperature is significantly lower than the internal body temperature

The nature of generalized lesions can be studied in lesions arising after intravenous inoculation. While this experimental device for the production of generalized lesions may not precisely imitate the manner of the distribution of treponemes, the resulting lesions appear to be identical The distribution of lesions after intravenous inoculation has been described, particularly by Chesney & Schipper.[13]

Hollander & Turner [29] examined the influence of temperature on the development of lesions after intravenous inoculation. In repeated experi-

TABLE XIV DEVELOPMENT OF SYPHILITIC LESIONS AFTER INTRADERMAL INOCULATION OF THE EARS OF NORMAL AND SYMPATHECTOMIZED RABBITS *

Experiment number	Rabbit number	Number of treponemes in inoculum	Site	Back lesions (days)	Normal ears		Sympathectomized ears	
					Lesions (days)	Mean temperature (°C)	Lesions (days)	Mean temperature (°C)
I	25-86	500	Ears		—0—	29		
	25-87	500	„		27	26		
	25-88	500	„		18	21		
	25-89	500	„		27	29		
	25-92	50 000	„		10	28		
	25 93	50 000	„		—0—	26		
	25 94	50 000	„		10	25		
	25 95	50 000	„		—0—	27		
	25 90	500	Back	15		23		
	25-91	500	„	15		23		
II	26-42	500	Ears		—0—	24	29	30
	26-43	500	„		—0—	26	31	30
	26-45	500	„		—0—	24	21	30
	26-46	500	„		—0—	27	20	30
	26 59	500	„		—0—	26	20	34
	26-62	500	„		—0—	23	18	33
	26-63	500	„		—0—	26	20	31
	26 71	500	„		—0—	22	32	29
	26-48	500	Back	15		22		22 [a]
	26-58	500	„	15		21		21 [a]

* From Hollander & Turner [illegible]
—0— = no lesions developed
[a] Sympathectomy regarded as unsuccessful

ments, of which representative animals are illustrated in Fig 4, it was demonstrated that the treponemes preferentially localized in the cool areas of the body. The shaved surface of the rabbit's back, in the cool-room, regularly seemed to offer the optimum conditions, and at the same time contrasting areas, protected by fur and therefore slightly warmer, did not develop lesions.

In a variation of this experiment (Table XV), areas of skin were shaved well before the inoculation, in order to minimize any element of trauma to the tissue, since, as shown by Chesney et al. [12, 14, 24] traumatized areas may provide a favorable site for growth of treponemes It was concluded from the results of this experiment, summarized in Table XV, that trauma

FIG 4. LOCALIZATION OF LESIONS IN SHAVED AREAS OF RABBITS AFTER INTRAVENOUS INOCULATION OF "T. PALLIDUM"



These rabbits (Nos 38-85 and 38-86) were inoculated intravenously with 1 0 ml of an

played no role, and that temperature was, in fact, the controlling influence in this situation. Additional evidence pointing to the role of temperature was provided by the observation that development of lesions in shaved areas could be suppressed by maintaining the animals in a warm-room.

TABLE XV THE LOCALIZATION OF LESIONS IN SHAVED AREAS AFTER INTRAVENOUS INOCULATION OF 10 000 000 TREPONEMES OF THE NICHOLS STRAIN *

Environment	Rabbit number	Day of shaving each quarter of back in relation to day of inoculation			
		−16 [a]	0 [a]	+7 [a]	+19 [a]
Cold (18°-21°C)	45 54	40	40	0	0
	45 55	40	40	0	0
	45 56	40	20	0	0
	45-57	15	20	1	0
Warm (29°-31°C)	45 50	0	0	0	0
	45 51	0	0	0	0
	45 52	?	?	?	0
	45 53	0	0	0	0

* From Hollander & Turner [86]

[a] Numbers signify number of skin lesions developing in the respective quarters of the rabbit's back

? = Few transient questionable lesions

Summary of temperature effects

In summary, it may be said that much of the variation in susceptibility of various tissues to infection with treponemes can be interpreted in terms of the restricted optimum growth-range of the treponeme superimposed upon the temperature gradients of the host The temperature at which treponemes ordinarily multiply *in vivo* is in the range of 30-38°C. The optimum temperature level is probably in the neighborhood of 35-37°C.

Treponemes, which do not seem to survive above 40°C, illustrate a principle stressed by Lamanna & Mallette:

" A curious phenomenon repeatedly observed throughout the living world is the tendency for optimum temperatures to be closer to the maximum than minimum temperature. In the case of the bacterial pathogens of warm-blooded animals the temperature of the natural habitat of the pathogen may be only a few degrees removed from the maximum temperature for growth. " [84]

The extent to which the experimental observations can be extended to treponemal disease in human beings is a matter of speculation. Local body temperature is, however, certainly a limiting factor and it may be permissible to wonder, for example, whether certain clinical peculiarities

of human disease, such as the usual delimitation of syphilitic aortitis to the thoracic aorta, may not be a reflection of a temperature gradient.

Finally it is interesting to speculate on temperature and its relation to the differences among treponemal species. It is general knowledge that yaws is largely limited to areas of the world in which the climate is warm and in which the mean temperature-range is small. But at this stage in our knowledge we can only guess at how much of the difference in the clinical pictures of the various treponematoses is due to temperature differences in the hosts as a result of their environments; or speculate on what effect the successive passages of treponemes for centuries, under the *influence of the tropics, has played in the development of distinctive species* of yaws treponemes.

Hormonal Influences

The normal progress of a treponemal infection has been presented in this discussion as the dynamic interaction between the multiplication and accumulation of treponemes on the one hand, and their destruction by the body defenses on the other. We have considered in detail the influence of temperature, which is one of the principal complicating factors directly affecting the growth of the treponeme The influence of hormones appears to be of a different character and operates by inhibiting the action of various body defenses, both specific and non-specific. Previous observations on the influence of sex hormones and the sex of experimental animals [11, 21, 30, 31, 37] suggest that the same type of response may be elicited by various hormones, although it is perhaps not justifiable to generalize from one type of hormone to another. In general, estrogens have an inhibiting action and androgens an enhancing effect. There is evidence too, from clinical data, that the disease in human beings is influenced in a similar manner, but none of these effects greatly alters the course of syphilis either in man or in the experimental animal

It has been demonstrated, however, that the adrenocorticosteroid hormones have a profound effect on the evolution of experimental treponemal infections. These effects were described first by Turner & Hollander,[55, 56] and have been confirmed in substance by numerous investigators.[16] Since the administration of cortisone and related compounds leads to alterations in experimental treponematoses which have both theoretical and practical implications, these studies will be summarized below.

Features of characteristic cortisone lesions

When cortisone is given to rabbits with syphilitic lesions in the early developmental stage there is a striking alteration in the evolution of the disease. In contrast to the usual course, described in Chapter 2, the lesions

quickly become globoid in configuration, and pale, soft and spongy, with a sharply circumscribed base They do not become indurated and do not progress to the ulcerative stage observed in the usual course of events (see Plate I(A)), but slowly increase in size, while remaining globoid in shape and sharply circumscribed (Plate I(B)).

When the interior of a cortisone lesion is examined it is found that the lesion is filled with a thick mucoid gelatinous-like material which oozes freely from the cut surface. Examination of this material under the dark-field microscope shows treponemes literally swarming over the entire preparation The same concentration of treponemes had never before been encountered in our laboratory in syphilitic lesions. The comparative counts observed in the initial cortisone experiment are recorded in Table XVI

TABLE XVI TREPONEME COUNTS ON SYPHILOMAS OF CORTISONE-TREATED AND CONTROL RABBITS *

Group	Rabbit number	Darkfield treponeme counts Number per 100 oil immersion fields		
		Post treatment day		
		Fifth	Seventh	Tenth
Cortisone treated [a]	29-63	3 000	2 960	2 300
	29 66	1 500	1 002	680
	29-67	750	406	1 650
Controls	29-62	116	27	7
	29-64	140	69	156
	29 65	224	35	3

* From Turner & Hollander [64]

[a] Inoculated with 500 *T pallidum* in each of 4 sites, cortisone 3 mg/kg body-weight twice daily for 10 doses started 25th day after inoculation

The evolution of cutaneous syphilomas was described in Chapter 2 The lesions are characterized at an early stage by the presence of mucoid material, which has been identified as hyaluronic acid As the evolution proceeds, there is a marked infiltration with mononuclear cells, and when ulceration supervenes there is often extensive necrosis with large numbers of polymorphonuclear cells

The miscroscopic appearance of the cortisone-treated lesion (Plate II(D), (E), (F)) is very different from that of the non-treated lesion (Plate II(A), (B), (C)), yet the reactions in the two instances are qualitatively similar The principal differences are that in the lesions of the cortisone-treated animal mucoid material is much more prominent, occupying most of the lesion, and the cellular reactions are much less prominent, being almost completely suppressed

The mucoid material is found between the epithelium and the muscle layer, and diffusely between the tissue cells both in the cortisone lesion and in the untreated lesion. In the former, however, it is not separated into lobules, but seems to have coalesced into a single mass. In both lesions the mucoid areas stain metachromatically with toluidine blue, but in the cortisone lesion these areas stain more intensely (Plate II (B), (E)).

Our associate, Dr Robert Millonig, has recently made quantitative determinations of the amounts of hyaluronic acid in cutaneous syphilomas from cortisone-treated and untreated rabbits Pooled syphilomas of untreated animals contained 1 mg of hyaluronic acid per gram of wet weight or 4.4 mg per mg of dry weight. Pooled syphilomas from cortisone-treated animals contained 3 mg per gram of wet weight or 14 1 mg per mg of dry weight The hyaluronic acid content of uninvolved skin of these untreated and cortisone-treated rabbits was essentially the same, being respectively 0 43 mg and 0 44 mg per gram of wet weight, and 0.85 mg and 1 0 mg per mg of dry weight [a]

The type and distribution of cells seen in the microscopic sections are also similar except that in the cortisone lesion there is merely a thin band of scattered lymphocytes about the periphery, while in the untreated lesion there are dense accumulations extending into the interior of the lesion Necrosis is also present in both lesions but again fewer acute inflammatory cells are seen in the cortisone lesion (Plate II (A), (D))

Cortisone time and method of administration

Cortisone has been administered usually in daily doses of 6 0 mg per kg of body-weight for periods of 1-4 weeks After administration of cortisone to rabbits during the incubation period, the syphilitic lesions appear at the same time as in the untreated control rabbits The absence of any influence on the course of the infection during this stage is in agreement with the hypothesis that the action of cortisone is primarily on the host rather than on the treponeme.

When, however, cortisone is given to rabbits with active early or subsiding lesions, a typical change in the nature of the lesion toward that of the cortisone type is observed. Cortisone administered at a time when the healing stage is well advanced induces no detectable modification of the progress of the healing process.

With long-continued administration of cortisone (4 weeks or longer) many animals appear to " escape " from the action of the drug Some begin to show regression and healing, while others exhibit an exacerbation of the

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[a] Earlier chemical studies carried out by Dr Frederick A H Rice in this laboratory (cited in reference 56) have recently been published in detail (Rice, F A H (1956) Chondroitin sulfate and hyaluronic acid in syphilomas of cortisone-treated rabbits, *Science*, 124, 275)

lesion similar to that seen in cortisone-treated animals at the end of treatment.

Upon the withdrawal of cortisone from animals with typical cortisone lesions a fairly characteristic change, which we have designated the "rebound", takes place in the lesions They become greatly enlarged, tense

FIG. 5. EXTENSIVE SYPHILITIC INVOLVEMENT OF BACK OF RABBIT FOLLOWING "REBOUND" AFTER CORTISONE TREATMENT



[illegible] present at the base of the ears, eyebrows, nose and scrotum

and erythematous, and have a considerable infiltration spreading out from their bases. The lesions macroscopically and microscopically resemble those in untreated animals, except that they are much larger. The rebound is an expression of the very large numbers of treponemes present in the lesions of cortisone-treated rabbits; a photograph of a rabbit with very extensive syphilomas developing after the withdrawal of cortisone is shown in Fig 5.

Penicillin in cortisone-treated animals

From the studies which are described in Chapter 6 it is known that penicillin in appropriate doses will effect a striking reduction in the treponeme count of cutaneous syphilomas in rabbits within 24 hours. This method was employed on 6 cortisone-treated rabbits and 2 controls, treponeme counts being made on the lesions before the penicillin was administered and 24 hours after the initial dose

Treponemes counts on the lesions 24 hours after the initial dose of penicillin showed a marked reduction in the number of motile treponemes in all animals, and, as is customarily observed, only an occasional treponeme—either motile or non-motile—in the control animals In the lesions of the cortisone-treated animals, however, in addition to an occasional motile treponeme, a very large number of non-motile (and presumably dead) *T pallidum* was present These observations have been repeatedly confirmed.

From this it appears that while penicillin is equally effective in killing treponemes in cortisone-treated and in non-cortisone-treated animals, the mechanism of clearing killed or damaged organisms from the lesions is less efficient in the cortisone-treated animals The presence of large numbers of non-motile treponemes in this situation suggests that the removal of dead treponemes is a function of inflammatory cells. However, the larger number of motile treponemes in the lesions before treatment may be partly responsible, and it is also suggested that the presence of copious amounts of mucoid material may interfere with the activity of the phagocytic cells

Discussion of cortisone effects

After preliminary experiments with cortisone reported in 1950, Turner & Hollander [55] stated that the influence of cortisone was "in a sense to produce a dissociation of parasite and host", a concept borne out by the more extensive experiments reported in 1952 [56] In addition, this idea is in accord with numerous observations that have been made on the effect of cortisone in other infections, particularly tuberculosis.[36, 60]

By "dissociation," we intended to imply that the body defenses were suspended while the treponemes kept growing without restraint Accordingly no effect is observed or is to be expected during the incubation period at a time before the body defenses become active. But when cortisone is given during the early stage of active lesions, the interference with the host defense allows the lesions to progress to a state which does not normally develop. In this state the lesions are essentially a pure culture of treponemes, without dead or dying organisms, without cellular infiltration, and possibly with some decrease in serum antibody. Finally, when an infection has progressed to an abnormal state under the influence of cortisone, and the cortisone is discontinued, the observed results are what would be expected

in the presence of an enormous number of organisms and a normally reacting host

This "rebound" or resurgence of the disease has been observed in many other infections.[22, 23, 36, 60]

The mechanism of cortisone action in isolating the treponemes from the host defenses is not known. At least part of the action may be through the inhibition of antibody formation; however, this could not be clearly demonstrated in our experiments During treatment with cortisone, some animals showed lower-than-usual antibody levels, while others showed very high levels.[56] The difference between these observations and the findings of Germuth,[22, 23] who demonstrated that cortisone can suppress the formation of antibody, appears to depend on both the amount of cortisone and the amount of antigen administered In contrast to the fixed amount of bovine albumin antigen used by Germuth in experiments in which antibody formation appeared to be suppressed, these rabbits received a living and exponentially increasing culture It was noticed that even the animals with the most evident cortisone effects eventually tend to "escape" from clinical restraint, after receiving cortisone for long periods Regardless of just what role the suppression of antibody formation may play in the disease pattern of cortisone-treated animals, the typical changes occur much too quickly following the initiation of cortisone therapy (58-72 hours) to be due entirely to changes in serum antibodies.

The mucoid material in the lesions is believed to be a product of the treponeme The principal evidence for this is the fact that this material accumulates in the cortisone-treated lesions, where large numbers of treponemes are also accumulating, and at a time when there are no cellular elements present which would be likely to produce it

An interesting question is the fate of this hyaluronic acid in the non-cortisone-treated animal, or in the treated animal after cessation of treatment Turner & Hollander [58] have suggested that it is normally converted into chondroitin sulfate, and that this conversion is the source of the indurated character of the usual lesion The subject is under further investigation at the present time

The administration of cortisone enables one to procure large numbers of treponemes in the laboratory for such purposes as immobilization tests, the preparation of agglutinating antigen, the study of specific immunologic components, or the examination of the peculiar mucopolysaccharide. For these purposes, testicular lesions are convenient since they are influenced by cortisone in the same manner as skin lesions.

In general it can be stated that all tested strains of treponemes—whether from cases of syphilis, yaws, bejel or endemic syphilis—are affected in much the same way as the Nichols strain of *T. pallidum*, although the deviations from the usual pattern may not be so striking Treponemes are considerably more numerous in the lesions of the cortisone-treated animals,

but the degree of accumulation of hyaluronic acid varies considerably from strain to strain. (See Chapter 7.)

Influence of Specific Antibody

The nature of the evolving immune-reaction of the host to treponemal infection will be examined in Chapter 5. For the moment we shall refer only to the interfering effects of specific antibody occurring incidentally on the recipient host, either as a result of prior infection with cuniculi treponemes or as a result of the transfer of small amounts of antibody along with the inoculum.

Prior cuniculi infection

The presence of cross-reacting antibody against *T cuniculi* may seriously interfere with the work of the experimental syphilis laboratory. Cross-immunity between syphilis and yaws on the one hand and cuniculi infection on the other was demonstrated by Turner, McLeod & Updyke [57] in 1947, and later the occurrence of a specific cross-reacting serum antibody was demonstrated by Khan,[32] and Khan, Nelson & Turner.[33] Rabbits obtained from commercial breeders often have had prior cuniculi infections. Such rabbits appear normal and healthy and the testes likewise appear normal, although sometimes careful scrutiny of the scrotal sacs will reveal characteristic, inconspicuous, stellate scars over one or both testes. Hardy (unpublished observations), and Hardy & Nell [25] found that sera from animals with these scars often give positive agglutination tests (TPA), and that when such animals are inoculated with large doses of treponemes for the preparation of agglutinating antigens the harvested spirochetes tend to agglutinate spontaneously. The spontaneous agglutination is believed to be a result of specific treponemal agglutinins remaining from the prior cuniculi infection to which has been added a rapid booster-type rise in antibody induced by the recent inoculation and multiplication of the new treponemes Although no series of similar rabbits has been tested with the immobilization test, it is likely that animals which have had cuniculi infection can also be identified by that test.

The presence of previous cuniculi infections probably provides the explanation of occasional and hitherto very puzzling " misses ", or animals which are very much more resistant to syphilis than the average The reaction of immune and partially immune animals to reinfection is discussed in more detail in Chapter 5.

Passive antibody, " sensitization "

Antibody may be transferred from the donor animal to the recipient by way of the inoculum. As Brown & Pearce [6] wrote in 1920 :

" Transfers made from actively developing lesions or from animal to animal as rapidly as the infection developed tended to produce or maintain a short incubation period, while

inoculations made from old, inactive or regressing lesions showed a relative prolongation of the incubation period irrespective of the dose of spirochetes used."

The effective number of treponemes in these inocula was apparently reduced by the accompanying antibody

Another manifestation of passively transferred antibody has been demonstrated *in vitro* by Kahn [32, 33] and others [41, 54] in this laboratory as the "sensitization" phenomenon.

The sensitization phenomenon can readily be demonstrated when treponemes for the immobilization test or for the agglutination test are obtained from old infections, and indeed sensitization is a frequent cause of unsatisfactory treponeme suspensions in these tests The phenomenon will be described more fully in Chapter 4; in brief, in the presence of antibody attached to the treponeme, there occurs a specific immobilization or agglutination, as the case may be It is not clear why the same phenomenon does not occur in the donor animal, two alternative explanations suggest themselves One is that specific antibody, which is present in the donor animals' serum, is not actually attached to the treponeme *in vivo*, but only becomes attached when tissue emulsions release antibody into mixtures. Another possible explanation is that even though antibody is attached to the treponeme there is insufficient complement present *in vivo* to potentiate the specific antigen-antibody reaction, and again only the disruption of cells in the preparation of the emulsion, or the intentional addition of complement, provides this essential component

Whatever the mechanism may be, it is probable that the sensitization phenomenon is an important factor in transfers of treponemes from animal to animal of the same species, or between animals of different species It is an observed fact that early treponemal lesions provide the best inocula, while older lesions, where antibody may already have formed, are less satisfactory This phenomenon may be the basis for the difficulty in recovering treponemes from tertiary syphilitic lesions in man, and it may also underlie our lack of success in isolating pinta organisms from patients, all of whom had long-standing disease

Miscellaneous Factors Influencing the Experimental Disease

Metals and antibiotics

Treponemes may encounter within the so-called normal animal body various substances such as metals and antibiotics, which inhibit or prevent their optimum growth Many of these substances have clear-cut therapeutic action, as is discussed in Chapter 6 But other substances less well known, and indeed as yet unidentified, may have significant anti-treponemal action

Levaditi & Lépine [35] examined 45 elements and found that 10 of these had some treponemicidal action As pointed out in Chapter 6, most of the

antibiotics now in clinical use have significant anti-treponemal action. To what extent these substances are being encountered inadvertently is not known, but their presence may at times explain strange and confusing experimental results.

Local tissue susceptibility

It was noted above that local tissue susceptibility is greatly influenced by the temperature of the particular tissue. Unfortunately, we have only scattered hints as to what other tissue-affecting conditions have controlling influences on the growth of treponemes With regard to the tissue requirements for treponemal growth, it should be noted that much of the experimental work has involved infection of the skin or testis. Clearly these are adequate tissues and just as clearly other tissues such as fat are inadequate, for lesions never seem to originate in them Possibly some required physical condition or some chemical ingredient is lacking in these tissues. One of the peculiarities of human treponemal skin lesions and of experimental yaws lesions in hamsters (see Chapter 2) is the tendency towards centrifugal extension of the lesion in an annular fashion, with central healing One suspects that some required ingredient may have become depleted behind the advancing margin; although the possibility of local immunity has not been ruled out

Anaerobiosis

While it is generally believed that pathogenic treponemes cannot survive in the presence of oxygen, the development of treponemal lesions in proximity to arterial blood casts some doubt on the antitreponemicidal action of oxygen *per se* The oxygenation of tissue is nevertheless probably a factor influencing the growth and multiplication of treponemes, directly or indirectly, but again this does not seem to have been verified by direct experimentation.

The studies of Fildes with the anaerobic tetanus bacillus will, however, serve as a model of what may be expected in the case of treponemes. Fildes [18] has stated that "There seems little reason to doubt that the main conclusions relating to *Bacillus tetani* may be applied in principle to all anaerobic organisms." Vaillard & Vincent [58] are given credit for the discovery, in 1891, that the spores of *B. tetani* do not grow *in vivo* except under special conditions Fildes [17] suggested that the normal oxygen tension of the tissues was unsuitable for the germination of spores, and that to allow germination a lower oxygen tension had to be provided. Most of the methods which were successful in stimulating the growth of tetanus spores *in vivo* reduced the oxygen tension locally by the production of tissue necroses. Fildes then demonstrated that a lower oxidation-reduction potential was in fact required for germination both *in vitro* [18] and *in vivo*.[18]

When the animal-tissue oxygen tensions of guinea-pigs were reduced to 8 mmHg by carbon monoxide, kept at the normal level of 29 mmHg, or raised to 38 mmHg by 60% O_2, Campbell & Fildes[10] produced 75%, 45% and 6% clinical tetanus respectively, by injecting spores plus aleuronate intraperitoneally.

It is also pertinent to examine the contrasting behavior of the aerobic organism *Mycobacterium tuberculosis*. Rich & Follis[50] have shown in guinea-pigs that inoculated tubercle bacilli are influenced by a change in the environmental oxygen tension At a tension of 10 volumes per cent, corresponding to an altitude of 19 000 feet (5791 m) significantly fewer and smaller lesions developed than in animals at normal atmospheric oxygen tension

A relationship of the tissue oxygen-supply to the susceptibility to infection with tuberculosis was first suggested by Corper, Lurie & Uyei[15] and has been discussed at length by Rich[49] The tissue susceptibility to syphilis is in a reverse order. A high oxygen-supply provides the most suitable environment for the propagation of tubercle bacilli, but is unfavorable for treponemes In the lungs, for example, tubercle bacilli find the best conditions, but treponemes can only rarely produce lesions Conversely where the oxygen supply is low, as in the human fetus, the tissue is markedly resistant to tuberculosis, but here may be the site of the most extraordinarily extensive syphilitic infection

The *in vitro* behavior of treponemes with respect to anaerobiosis and oxygen is discussed in Chapter 4

The necessity for the presence of a suitable oxidation-reduction potential may be the explanation of the curious relationship between experimental syphilis and vaccinia reported by Pearce & Murphy,[47, 48] who found that extremely large numbers of generalized lesions developed after either intratesticular or skin inoculation of treponemes, when a vaccinia infection was in progress The foci of vaccinia lesions may have provided the necessary anaerobic conditions required by the treponemes.

The same reasoning applies to the observations of Chesney and his co-workers[12, 14, 21] who observed that treponemes grew well in old granulating wounds, and noted, too, that such wounds, coal-tar dermatitis lesions, the testis, and the ectodermal cells of the skin, all provided suitable sites for treponemes, and that all these sites are characterized by the presence of actively proliferating cells In the light of our present knowledge it seems reasonable that the metabolism of these proliferating cells lowers the oxidation-reduction potential to the level required by the treponemes.

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Chapter 4

CHARACTERISTICS OF TREPONEMES IN VITRO

Since pathogenic treponemes cannot be cultivated successfully on artificial media, characterization of these organisms is limited by the fact that they cannot be studied entirely free of host tissue. Modern methods of extraction and differential centrifugation do yield relatively "pure" treponemes, but doubt is always present, particularly in chemical or immuno-chemical analysis, as to the role played by normal or modified host tissue.

Cultivated forms of spirochetes are available for study, and some of these strains morphologically resemble pathogenic treponemes; but again the question always arises as to what extent the characteristics of the cultivable forms can be considered to reflect those of the pathogenic variety.

Despite these limitations, however, much useful information has been accumulated on the biologic characteristics of pathogenic treponemes from *in vitro* studies

Experimental Methods

The *in vitro* work in this laboratory has been carried out mainly with emulsions of rabbit testes infected with the Nichols strain of syphilis Scattered observations with other strains of syphilis, yaws, and bejel treponemes suggest that most of these findings could be duplicated with any other strain. The Nichols strain readily produces prominent lesions in rabbits and has been used principally in the USA In European laboratories the Truffi and the Gard strains, which also produce prominent infections in rabbits, have been employed

Source of treponemes

The rabbit testis, which is one of the most suitable sites for the growth and multiplication of treponemes, is the most convenient source of treponemes for *in vitro* studies The large testes of mature rabbits offer a site which is not only favorable for the growth of treponemes, but also not

readily subject to secondary infection. If desired, one or both testes can be removed surgically from the anesthetized animal.

It should be stressed that, while the rabbit testis may be a convenient source of treponemes, it is not a simple matter to obtain large yields of treponemes relatively free from normal rabbit tissue. Indeed, it is unlikely that any method used at present can effect a separation of the rabbit tissue from the treponemes to a point where it cannot be detected immunologically.

It is almost as difficult to obtain suspensions of treponemes free from detectable amounts of specific antibody, yet this is an important objective when preparing antigens. The immobilization of "sensitized" treponemes by the addition of complement, and the spontaneous agglutination of sensitized TPA antigens, are the principal difficulties in accomplishing the TPI and TPA tests, respectively.

For the best results in terms of relatively pure suspensions of treponemes it is necessary to produce an overwhelming, rapidly progressive infection and to harvest the treponemes early. In order to do this, particular attention must be directed to the various factors discussed in the preceding chapter, which may adversely influence the development of the infection.

A large inoculum is required, for the orchitis seems to be most satisfactory when the incubation period is very short. It may be advantageous to make repeated transfers from rabbit to rabbit at short intervals in order to obtain progressively shorter incubation periods and progressively greater inocula, and cortisone may be used to prolong the period during which treponemes accumulate, and to increase the yield of organisms. In practice it is convenient to prepare a large volume of source material containing something like 100 000 000 or more treponemes per ml. This should be divided into small portions and preserved by freezing at -70°C, for use as needed. Because treponemes survive so poorly outside the animal, particular attention must be paid to the suspension, in order that the maximum number of treponemes should remain viable. A 10% solution of normal inactivated rabbit serum in physiologic saline with 15% by volume of glycerol is a satisfactory diluent which allows freezing if required. It is necessary, of course, that "normal" serum be obtained from rabbits which have not been naturally infected with *T. cuniculi*. Complicated "survival" media containing thioglycollate are not recommended for the preparation of suspensions intended for inoculation.

For the production of good treponeme suspensions, mature, healthy animals which have not been previously infected with *T. cuniculi* are essential. They should be maintained in a cool environment and they must be given food which does not contain antibiotics.

To minimize the "sensitization" of treponemes and the accumulation of specific antibody the testes must be removed soon after the appearance of lesions. Where very large yields of treponemes with little or no antibody

are desired, as for example in the preparation of agglutinating antigens, cortisone may be employed with advantage. Large numbers of treponemes with relatively little antibody have also been obtained, by other investigators, with the use of nitrogen mustard, and after total body irradiation.

Extraction

Suspensions are commonly prepared from infected testes by extraction in saline, serum saline, or in modified thioglycollate media with agitation in a 30-35°C water bath Before extraction the testes may be sliced or minced with scissors, or they may be ground in a mortar, with or without sterile sand, after which the sand and coarse particles of rabbit tissue can be removed by light centrifugation.

Hardy & Nell[19] recommend exsanguination of the animal in order to reduce the amount of antibody removed with the treponemes. The use of citrate and centrifugation in the cold at 4°C—as in the extraction method of Hardy & Nell—possibly serves the same purpose Sausse, Borel & Hardy[47] have indicated that antibody production can be retarded by the repeated injection of rabbits with an antisyphilitic-rabbit-globulin sheep serum.

Enumeration

Much of the recent knowledge of treponemal infection has been obtained with the help of efficient reproducible counting methods In this laboratory quantitative enumeration of treponemes has been applied to such diverse subjects as the rate of multiplication of treponemes *in vivo*, the evaluation of antibiotics and drug action, and the relationship of environment to the course of infection These investigations are described in other sections

The general method for the enumeration of treponemes has been discussed by Bessemans & De Moore,[1] by Morgan & Vryonis,[29] and by Magnuson, Eagle & Fleischman[27] In this laboratory the routine method of counting requires 0 005 ml of suspension evenly distributed beneath a 22-mm-square coverslip The quantity is measured from a 0.2-ml Kahn-type serological pipette The number of treponemes counted in about 50 oil-immersion darkfields, multiplied by a factor of 100 000 gives the approximate number of treponemes in 1 ml of suspension The precise number of fields to be counted depends on the size of the microscopic field and must be calculated for individual optical systems

Gelperin,[13] in this laboratory, also obtained reproducible counts of culture treponemes by indirect methods Phototurbidimetric, Micro-Folin and Kjeldahl methods were satisfactory, but because of errors introduced by uncontrolled amounts of tissue in the suspension, these methods are not readily adaptable to the enumeration of treponemes derived from living tissue

Physical Characteristics of Treponemes

Morphology

The most accurate contemporary measurements of *T. pallidum* by means of electron microscopic visualization by Swain [59] and others differ little from those made by Noguchi,[38] 37 years ago (Table XVII). The organisms are actually much thinner than is often appreciated, and although their length is about twice the width of a human red blood cell, the total volume of protoplasm is extremely small.

TABLE XVII DIMENSIONS OF TREPONEMES BY OPTICAL MICROSCOPE (NOGUCHI) AND ELECTRON MICROSCOPE (SWAIN)

Dimensions	Noguchi [38]	Swain [59]
Length	8-14 μ	6-15 μ
Width	0.25-0.3 μ	0.09-0.18 μ
Wave length of spiral	1 μ	1.15 μ

The fact that there is altogether such a small amount of protoplasm may account for much of the difficulty in the visualization of the treponemes either alive or after fixation and staining. It is pertinent that successful methods of staining treponemes have usually required the deposition of dye or metallic particles on the surface of the organisms, and this fact may account for the thickness value given by Noguchi, who was measuring stained preparations It is a tribute to the persistance and skill of the early observers, beginning with Schaudinn and Hoffmann in 1905, that not only stained treponemes but also living motile organisms were visualized without the benefit of darkfield or phase-contrast illumination

Ordinarily the thinness of the organisms precludes their visualization in the light microscope, unless the contrast is increased, either by a darkfield condenser, or by indirect illumination, or after fixation and staining.

The electron microscope has brought new opportunities to the study of the morphology of treponemes It should be noted that, unlike the refinements of phase and darkfield optical microscopy, which merely increase contrast, the electron microscope actually increases the resolution

The earliest electron microscope pictures [30, 31, 72, 73] showed flagella-like structures which had not been seen before. With improvement in techniques of shadow casting these structures have been demonstrated with

increasing clarity. In addition, it has been noted, first by Watson et al.[68] that these flagella-like structures occur in a bundle resembling an "axial filament" about which the treponeme is wound. Examination and comparison of the published electron photomicrographs show that a structure suggesting an axial bundle is present in those preparations in which the spirals are intact, while in preparations in which the spirals are distorted and separated, flagella-like structures, attached at only one end, are seen. It may be recalled that one of the characteristics of *T pallidum* is the tendency for the spiral form to be maintained The inference is that the appearance of "flagella" in the distorted preparations is an artefact, and that these fibers arise from a bundle which normally serves as an axial filament Hampp, Scott & Wyckoff[18] demonstrated an axial structure in preparations of small dental treponemes and in cultures of Noguchi strain treponemes Bradfield & Cater[4] noted an axial structure in various species of spirochetes, and commented on its functions. Schaeffer, in this laboratory (unpublished observations), found that in some preparations of *T. pallidum* after wet fixation and staining with Schiff's reagent this structure could be seen with phase-contrast illumination The treponemes appeared to consist of two interwoven filaments, one stained and one unstained. It was not possible to produce this appearance consistently.

The final demonstration of this internal organization in pathogenic treponemes has been shown independently in beautiful electron pictures published by Von Schmerold & Deubner[51] and Swain[59] These three investigators have stressed the necessity for careful fixation to avoid disruption of the filaments Von Schmerold & Deubner describe the axis as a bundle of three "fibrils" with diameters of 0 02 μ

It is interesting to recall that in 1923 Zuelzer,[77] who had worked with many types of spirochetes, affirmed that an axial filament was characteristic of a large group of organisms, and that although it could not be demonstrated with treponemes or leptospira its existence could be predicted This belief, based on a study of the comparative morphology and motility of these and related organisms, was later accepted by Noguchi[39] Noguchi described the organism as follows "The essential structure of a treponeme is a spring-like axial filament and a layer of contractile protoplasm enclosed in a delicate periplast"

The existence of a "periplast" or a capsular structure surrounding the internal substance of the treponemes is supported by the photographs published by Swain,[59] and is very clearly demonstrated in those presented by Von Schmerold & Deubner.[51] Hampp, Scott & Wyckoff[18] had already published similar pictures of formaldehyde-fixed cultured treponemes and mouth treponemes Probably the failure of other photographers to demonstrate this outer layer is related to more destructive methods of fixation Von Schmerold & Deubner used alcohol, and Swain used osmic-acid fixation

Balloon-like structures in old cultures of the Reiter treponeme, which were described by Gelperin[13] with the darkfield and demonstrated by Rose & Morton[45, 46] with the electron microscope, require further clarification. These structures appear to be abnormal forms produced as the result of an unfavorable environment. *It is interesting to speculate upon whether they may originate through the failure of a capsular structure to* grow and divide while the organism itself is still growing. There is at present no convincing evidence that such structures occur with pathogenic strains of *T. pallidum*, or that they are stages of an orderly life-cycle, as suggested by De Lamater, Wiggall & Haanes.[9, 10]

Motility

The movements of treponemes are most simply described by again quoting Noguchi:[39] "In a fluid medium the movement of these organisms is rotatory, in a semisolid medium it is corkscrew-like." The nature of these movements has not always been appreciated. Differences due to density of the medium have been described as characteristic species differences, and corkscrew-progression has been erroneously described as a movement of "translation".

The corkscrew type of motility is characteristically seen in the direct darkfield preparations from early lesions, or from lesions of cortisone-treated animals, which contain a large amount of hyaluronic acid. The rotatory type of motility is usual in serous exudates ordinarily obtained from lesions. We have observed that the corkscrew type of motility can be converted into the rotatory form when the mucoid material is thinned by hyaluronidase, and that the rotatory movement can be converted into the corkscrew type by the addition of methyl cellulose.

The corkscrew type of movement may be considered the normal method of locomotion, while the rotatory type of movement may be compared with the ineffectual racing of an aeroplane motor when the density of the air decreases at high altitudes, or with the racing of a marine engine when the screw rises above the water.

We have noted that Noguchi[38] argued for the existence of an axial filament in treponemes partly on the basis of their characteristic motility

"It is evident that in the treponemes no external flagellar apparatus is present, nor is any necessary. The axial filament, however, has several characteristics in common with bacterial flagella—*similar morphology and staining properties*, similar function, i.e.,

Staining

It is often stated that treponemes are difficult to stain On the contrary, treponemes are easily stained with any of a number of dyes. Yamamoto[29, 74, 75] was successful in staining treponemes with 402 different dyes out of 1315 which were examined Usually, because the organism is very small, little contrast is obtained, and the treponemes remain invisible unless they are in a relatively pure suspension with a clean background

The silver-staining method of Levaditi, as well as its many modifications and variations, increases the contrast by the deposition of metallic silver on the surface of the treponemes Unfortunately it is much more difficult to control the irregular deposition of silver in fixed tissue sections than in photographic emulsions, and silver stains often give variable and unpredictable results. Much of the extensive literature on staining has been reviewed by Campbell & Rosahn.[6]

The most recent addition to the long list of staining methods is the use of a complex dye (No. 3390-ink blue ASB diaminostilbene disulfonic acid coupled to 2 moles of Chicago acid, Parker Pen Co , Ganesville, Wisconsin) This dye was originally found by Gomez[18] in " Parker 51 " blue-black fountain-pen ink

Treponemes appear to be composed of at least three distinct morphologic structures to which staining techniques might be applied. the body, its intertwined axial filament, and the capsule, all of which were discussed in the section on morphology (see page 98) It was noted that Schaeffer, in this laboratory (unpublished observations), had, in isolated instances, been able to distinguish the first two of these structures as stained and *unstained, using wet fixation and Schiff's reagent The third structure is* the capsule suggested by electron pictures, or the " periplast " in the terminology of Noguchi The possibility that this structure contains the hyaluronic acid which accumulates so strikingly in cortisone-treated animals requires further study. Turner & Hollander[66] suggested in 1953 that the amount of hyaluronic acid produced by different species and strains of treponemes might be a specific characteristic of the species or strain Perhaps the structure and staining character of the mucopolysaccharide may vary from strain to strain At any rate, Yamamoto[74, 75] has stated that with two dyes, Acid Blue BBX (BA) (carbolized) and Alkali Blue B (SB) (5% carbolic acid added), he was able to stain *T pallidum* and *T pertenue* but not *T cuniculi*

Specific gravity and refractive index

Despite the use of the centrifuge for the preparation of treponeme suspensions, and despite the theoretical possibility that differential centrifugation might be the basis for a very efficient system of purification there

TABLE XVIII RESULTS OF SEDIMENTATION OF AN EMULSION OF TREPONEMES IN 20% RABBIT SERUM IN BROTH FOR 20 MINUTES AT VARIOUS SPEEDS IN AN INTERNATIONAL CENTRIFUGE No 2 *

Speed	Supernate	Sediment
Not centrifuged	++	++
2000	++	+++ definite concentration
4000	+ few	++++
4500	± occasional	++++
5000	0 one seen	++++
6000	0 none	++++
8000	0 none	++++

* *Turner & Kluth, 1941, unpublished data*

is little published information on the subject Consideration of the laws which govern sedimentation [15, 36] suggests that the peculiar shape of treponemes might be utilized in their purification, since irregular particles are sedimented relatively more slowly as the viscosity increases The purification of tobacco-mosaic virus by Stanley seems pertinent, and perhaps also the deduction of Debye & Bueche,[8] from an analysis of centrifugation data, that certain polymerized molecules have a coiled-spring shape

The general behavior of treponemes in ordinary suspensions upon centrifugation can be illustrated by some unpublished observations of Turner in 1941 (Table XVIII) In this experiment apparently all the treponemes were sedimented at 5000 r.p m and at higher speeds. In accord with this, in other experiments infectivity tests were always negative on supernates after centrifugation at 6000 r p m or at higher speeds.

The difference between the refractive index of the treponeme and that of the suspending media is the basis for the darkfield microscopic visualization of treponemes, yet again we have only limited data on the absolute values of this property. Nevertheless, herein may lie the explanation for a number of miscellaneous observations and impressions which require further study. For example, it has been suspected that some strains are more difficult to see than others, and that treponemes in suspensions containing hyaluronic acid, or glycerol, are easier to see than those in saline suspensions.

Survival of Treponemes at Ordinary Temperatures

Nelson and his associates working in this laboratory were largely responsible for developing methods for prolonging the survival time of treponemes *in vitro*. Nelson & Steinman [35] first demonstrated that the

anaerobic thioglycollate medium of Brewer,[5] which Eagle & Steinman[12] had found suitable for the growth of the Reiter-cultured strain of treponemes, could be adapted for pathogenic treponemes. In this media and its subsequent modifications, recommended by Nelson,[32, 34] pathogenic treponemes retain their motility for relatively long periods The tremendous improvement of this medium, over prior methods of prolonging survival, opened the door for the development of the treponemal immobilization test,[34] which was a distinct improvement over the cumbersome neutralization test with rabbits.[26, 61, 67]

TABLE XIX SURVIVAL MEDIA FOR "T PALLIDUM"*

Media	1948 Nelson [32]	1949 Nelson [34]	1950 Nelson [33]	1953 Weber [65]
Bovine serum fraction V or crystalline bovine albumin	0 00057M	0 00028M	2 00 %	2 g/100 ml
Na_2HPO_4 KH_2PO_4	0 010M 0 004M	0 010M 0 0038M	0 36 % 0 05 %	0 4 g/100 ml 0 05 "
Sodium thioglycollate Cysteine L(+) HCl Glutathione	0 0008M 0 001M 0 001M	0 0016M 0 001M 0 001M	0 03 % 0 03 % 0 02 %	0 05 g/100 ml 0 02 " 0 06 "
Sodium pyruvate Sodium chloride Serum ultrafiltrate	0 001M + +	0 001M + 0	0 01 % + 5 0 %	0 025 g/100 ml + 10 0 ml/100 ml
Vitamins	+[a]	+[a]	0	5 0 ml[b]
Sodium bicarbonate	0 0087M	0 0072M	0 06 %	0

* Devised by Nelson,[32-34] and Weber [65]

[a] Thiamin and niacin 1000 μg/l each, calcium pantothenate, pyridoxine, riboflavin, choline, inositol 500 μg/l, biotin and folic acid 10 μg/l final concentrations

[b] Supplement containing 14 vitamins This medium also contained small amounts of sodium citrate, tyrosin, tryptophane, gelatin hydrolysate, calcium chloride, and indigo disulfonate

The survival medium described by Nelson[32] in 1948 contained crystalline bovine albumin, phosphate buffer, sodium thioglycollate, cysteine, and glutathione, sodium pyruvate, sodium bicarbonate, sodium chloride, vitamins, and serum ultrafiltrate (see Table XIX) Anaerobiosis was obtained with the use of a Brewer jar which was evacuated and refilled with

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Speed	Supernate	Sediment
Not centrifuged	++	++
2000	++	+++ definite concentration
4000	+ few	++++
4500	± occasional	++++
5000	0 one seen	++++
6000	0 none	++++
8000	0 none	++++

* Turner & Kluth, 1941, unpublished data

is little published information on the subject. Consideration of the laws which govern sedimentation [15, 36] suggests that the peculiar shape of treponemes might be utilized in their purification, since irregular particles *are sedimented relatively more slowly as the viscosity increases* The purification of tobacco-mosaic virus by Stanley seems pertinent, and perhaps also the deduction of Debye & Bueche,[8] from an analysis of centrifugation data, that certain polymerized molecules have a coiled-spring shape.

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TABLE XX PYRUVATE REQUIREMENTS OF TREPONEMES FOR SURVIVAL "IN VITRO"*

Concentration of Na pyruvate	% Motile treponemes after days			
	4	8	12	16
0 0 %	0	0	—	—
0 01 %	90	70	52	8
0 025 %	90	82	50	20
0 05 %	92	86	32	16
0 1 %	92	84	48	12
0 25 %	94	70	28	8
0 5 %	74	28	8	0

* From Weber [69]

The rate of survival, observed by Weber [69] when pyruvate was added in increasing amounts, is recorded in Table XX.

Weber in disagreement with Nelson [32 35] found that bicarbonate and gaseous CO_2 were not required in the medium, and that their presence was not beneficial. Weber, however, could not obtain satisfactory survival without ultrafiltrate of serum, or its active crystalline factor isolated from bovine serum by Rice & Nelson [44] in this laboratory. The nature and function of this substance remains unclear

Weber also re-examined the relationship of temperature to the survival of treponemes *in vitro* In accordance with theoretical expectations, survival was found to be progressively shorter between 20°C and 40°C as the temperature was increased (See Table XXI)

TABLE XXI SURVIVAL OF TREPONEMES "IN VITRO" AT VARIOUS TEMPERATURES*

Incubation temperature (°C)	% Motile treponemes after days				
	4	8	12	16	24
20	95	94	82	82	22
25	97	83	54	25	6
30	95	71	44	11	0
35	75	22	2	0	0
40	0	0	—	—	—

* From Weber [69]

95% nitrogen and 5% carbon dioxide. In later modifications [33, 34] designed chiefly for the TPI test, the vitamins were omitted, fraction V was substituted for crystalline bovine albumin, and minor changes were made in the concentrations. For details of the preparation of the medium the papers of Nelson [32, 34] should be consulted.

Under certain circumstances much simpler media than the usual TPI survival media serve the same purpose Boak & Miller [2] reported that for immobilization 50% inactivated rabbit serum in saline can be used instead of the more complicated medium

The constituents of survival media have been studied by Weber [69] in this laboratory (see Table XIX) The primary function of the medium and of the manipulations which accompany its use appears to be the maintenance of an anaerobic state Hardy & Nell (unpublished observations) have noted that unsatisfactory immobilization tests are often associated with failure of the medium to maintain a low O-R potential Weber [69] found that 2.0 mg per 100 ml indigo disulfonate in the media was a convenient indicator of a satisfactory reduction level. A reduced state of the dye was ordinarily correlated with good survival

Evidence presented by Weber, however, indicates that the situation may be much more complex than the mere maintenance of anaerobic conditions, for although thioglycollic acid, cysteine, glutathione, or mercaptosuccinic acid, either singly or in combination, could be substituted for the reducing agents of the routine media, related substances such as cystine, oxidized glutathione, mercaptoethanol, ethanethiol, thiolacetic acid, ascorbic acid, sodium sulfhydrate, and sodium hydrosulfite were ineffectual, even when comparable anaerobic levels were obtained. Weber has suggested that active sulfhydryl compounds may be directly concerned in the metabolism of the organism Alternatively, it is possible that sulfhydryl groups play a dual role, one in establishing an effective anaerobic state and the other in neutralizing trace metals. The latter role was stressed by Brewer.[5]

Weber [69] obtained reproducible anaerobic conditions together with consistently good survival by using individual tubes in which the medium was layered with paraffin oil containing a powerful anti-oxidant (2, 6-di-tert-butyl *p*-cresol) This technique was devised to furnish a barrier against the entrance of atmospheric oxygen. In Brewer's thioglycollate medium, diffusion of oxygen is inhibited by the use of agar in the medium to increase the viscosity and reduce convection currents In the media advocated by Nelson the added bovine albumin may have this function in addition to its action as a " protective colloid " Hardy & Nell (unpublished observations) have found that gelatin can be substituted for fraction V of bovine albumin and may be preferable, since some batches of fraction V are relatively [illegible] for survival The difficulty with the fraction V may arise in a

medium

TABLE XXII IMMOBILIZATION OF TREPONEMES "IN VITRO" BY COMPLEMENT "SENSITIZATION" PHENOMENON *

Experiment number	Kind and source of treponemes			Material added			
	Organism	Testis	Day of orchitis	Saline [a]	Serum ultra-filtrate [a]	Normal rabbit serum [a]	Immune rabbit serum [a]
I Rabbit No 26-37	*T pertenue* Khan [14] (Table 8) Khan et al [14] (Table 3)	Left	1	88/82	82/80	80/82	0/72
		Right	6	50/88	48/92	44/92	8/88
II Rabbit No 26-44	*T cuniculi* Khan [14] (Table 8)	Left	1	80/78	80/84	80/82	8/80
		Right	12	36/83	35/80	40/76	8/92
III	*T pallidum* Nelson & Diesendruck [18] (Table 6)	Left	2	88/84	88/86	88/88	0/88
		Right	8	6/72	10/94	12/90	0/80

* From Khan,[14] Khan, Nelson & Turner,[15] and Nelson & Diesendruck[18]

[a] Numerator denotes the percentage of motile treponemes after 18 hours in a tube containing guinea-pig serum as a source of complement The denominator is the percentage of motile treponemes in a parallel tube containing an equal volume of heat-inactivated guinea-pig serum

TABLE XXIII TITERS OF A STANDARD POOL OF ANTISYPHILIS SERA MEASURED AGAINST SUSPENSIONS OF TESTES AFTER INCREASING PERIODS OF INFECTION *

Day of infection	Untreated animals		Animals treated with nitrogen mustard	
7	351[a]	309	363	328
7	450	417		
8	357	390	314	303
8	435	465	330	368
9	512	550	333	330
9	571	577	385	342
11	729	695	339	370
13	953	870	424	392
15	1075	1000	406	424
18	1199	1220	423	450

* From Seldeen[33]

[a] Titers are reciprocals of serum dilutions which would immobilize 50% of the treponemes

Originally, " the study of factors which influence the survival of *T. pallidum in vitro* was undertaken as a rational approach toward cultivation, in the hope that the attainment of conditions optimal for survival would lead to growth and multiplication " [32] Although improved survival has contributed notably to our knowledge of immunity in treponemal disease by furnishing a tool for the study of specific antibody, it has not led to the *in vitro* cultivation of treponemes, and it seems clear that the conditions for growth and multiplication are more exacting than the conditions for survival, indeed, in some respects, they may be altogether different Survival time can, in fact, be extended by factors which interfere with metabolism and growth. This is well illustrated by the prolonged survival which is possible at low temperatures. Obviously this is not a step toward growth and multiplication

Turner & Diseker [61] studied the special problems of the survival of treponemes under the conditions of storage which are likely to occur in blood-banks. It was found that under these conditions there was a progressive deterioration of treponemes. No infectivity could be demonstrated after 72 hours, and it was concluded that there was probably no risk of transmitting syphilis after storage for 4 days or longer. These observations and conclusions were substantiated in essence in similar studies by Bloch,[3] who found an emulsion infective after 72 hours, but not after 96 hours; but Selbie [52] found that a suspension of treponemes in rabbit plasma was still infective after 6 days at 5°C. The related problems encountered when blood or plasma has been frozen are discussed in connexion with survival at low temperatures

The Sensitization Phenomenon

Emulsions of treponemes prepared from long-standing animal infections always seem to be accompanied by a certain amount of specific antibody derived from the donor animal. The influence of this antibody on the course of infection in inoculated animals was discussed in Chapter 3. The *in vitro* manifestation of this antibody has been reported from this laboratory as the " sensitization " phenomenon

Khan,[24] and Khan, Nelson & Turner [25] reported that during the perform- [illegible] tests with *T. pertenue* and *T. cuniculi* the suspensions with-

" Since the same normal controls (normal rabbit sera, human serum ultrafiltrate, and

medium. "

details were reported by Turner & Fleming[65] working with treponemes, and Turner & Brayton[63] working with relapsing fever spirochetes

These early studies established that suspensions of treponemes remained infective for long periods when frozen and stored at the temperature of dry ice. In Table XXIV are listed examples of strains which were recovered from

TABLE XXIV VIABILITY OF TREPONEMES AFTER MAINTENANCE AT APPROXIMATELY —70°C FOR LONG PERIODS

Strain	Date frozen	Date thawed	Interval	Number of rabbits positive [a]
Cuniculi A	16 5 42	19 2 46	3 years 9 months	1/1
Cuniculi A	12 3 42	19 2 46	3 years 11 months	3/3
Cuniculi B	22 10 41	20 2 46	4 years 4 months	4/4
Nichols	22 3 42	20 9 46	4 years 6 months	2/2
Nichols	26 3 41	3 4 46	5 years	1/1
YD	2 4 40	21 5 46	6 years 1 month	1/3
Nichols	14 9 40	17 10 46 25 10 46	6 years 2 months	6/6
S6	1 11 41	6 1 48	6 years 2 months	1/1
C J	15 11 39	2 5 46	6 years 5 months	2/2
M J	15 5 41	17 12 47	6 years 7 months	1/1
M S I	26 2 40	21 10 47	7 years 8 months	1/1
M S II	26 2 40	21 10 47	7 years 8 months	2/2
S10	28 2 40	6 1 48	7 years 10 months	1/1
A G	21 12 39	17 12 47	8 years	2/2
M S I	26 2 40	22 6 48	8 years 4 months	2/2
LW	26 1 40	22 6 48	8 years 5 months	2/2
YC	24 5 40	1 10 48	8 years 4 months	1/2
YA	26 2 40	1 10 48	8 years 7 months	2/2
YH	5 1 40	1 10 48	8 years 9 months	0/2
YH	17 4 40	13 5 49	9 years 1 month	1/2
S10	28 2 40	13 5 49	9 years 1 month	1/2

[a] Numerator = number positive, denominator = number inoculated

the frozen state after intervals of 3 years and 9 months to 9 years and 2 months, during the period of the Second World War Although later attempts to recover frozen pre-war strains have been unsuccessful (Table XXV), this does not imply that the limit of survival times has been approached. Actually, numerous factors may have contributed to the poor recovery rate in 1949 For instance, during some preceding period the

Khan[24, 25] compared the amount of immobilization induced by complement after 18 hours in the alternate testes of a rabbit inoculated with *T. pertenue* on the 1st and 6th days of orchitis and in those of another rabbit inoculated with *T cuniculi* on the 1st and 12th days of orchitis. Nelson & Diesendruck[33] reported a similar experiment on the alternate testes of a rabbit inoculated with *T. pallidum*-and examined on the 2nd and on the 8th day of orchitis. In these experiments (see Table XXII), when the left testes were removed on the 1st or 2nd day of orchitis, immobilization by complement did not occur unless immune serum was added. When the right testes of the animals were removed after 6 or more days of orchitis, however, immobilization was observed not only with added immune serum but also in controls containing complement plus normal rabbit sera, ultrafiltrate of human sera, or saline.

Seldeen[53] measured the titer of a standard pool of antisyphilis rabbit serum against a series of treponeme suspensions prepared from testes after various periods of orchitis (see Table XXIII). There was a progressive increase in the serum titer when treponemes were removed from testes after increasing periods of infection. It is believed that the increasing deviation from the true titer represents an error introduced by the presence of antibody passively transferred from the infected testes When treponeme suspensions were prepared from a similar series of animals given nitrogen mustard a much lesser degree of variation was observed. This was interpreted to be a result of the inhibition of antibody formation by the nitrogen mustard.

Subsequently a large amount of evidence has accumulated in various laboratories performing immobilization tests which confirms the fact that it is necessary to use early infections in the preparation of immobilization-test suspensions, in order to avoid "sensitization" Indeed, one of the principal difficulties with both the immobilization and the agglutination procedures is the need for the respective antigen to be virtually free from passively transferred antibody

A parallel situation is known in other diseases Weinman,[70] for example, has discussed the difficulty which is present when one attempts to culture [illegible] from the blood of chronically infected animals He has [illegible] these conditions, [illegible] ice with a strong complement-inhibiting action.

Survival at Low Temperatures

Methods of preserving treponemes in the frozen state have been studied for almost 20 years by the senior author and his associates In 1938 Turner[60] first reported that treponemes could survive freezing and storage. Later,

details were reported by Turner & Fleming[65] working with treponemes, and Turner & Brayton[63] working with relapsing fever spirochetes.

These early studies established that suspensions of treponemes remained infective for long periods when frozen and stored at the temperature of dry ice. In Table XXIV are listed examples of strains which were recovered from

TABLE XXIV VIABILITY OF TREPONEMES AFTER MAINTENANCE AT APPROXIMATELY —70°C FOR LONG PERIODS

Strain	Date frozen	Date thawed	Interval	Number of rabbits positive*
Cuniculi A	16 5 42	19 2 46	3 years 9 months	1/1
Cuniculi A	12 3 42	19 2 46	3 years 11 months	3/3
Cuniculi B	22 10 41	20 2 46	4 years 4 months	4/4
Nichols	22 3 42	20 9 46	4 years 6 months	2/2
Nichols	26 3 41	3 4 46	5 years	1/1
YD	2. 4 40	21 5 46	6 years 1 month	1/3
Nichols	14 9 40	17 10 46 25 10 46	6 years 2 months	6/6
S6	1 11 41	6 1 48	6 years 2 months	1/1
C J	15 11 39	2 5 46	6 years 5 months	2/2
M J	15 5 41	17 12 47	6 years 7 months	1/1
M S I	26 2 40	21 10 47	7 years 8 months	1/1
M S II	26 2 40	21 10 47	7 years 8 months	2/2
S10	28 2 40	6 1 48	7 years 10 months	1/1
A G	21 12 39	17 12 47	8 years	2/2
M S I	26 2 40	22 6 48	8 years 4 months	2/2
LW	26 1 40	22 6 48	8 years 5 months	2/2
YC	24 5 40	1 10 48	8 years 4 months	1/2
YA	26 2 40	1 10 48	8 years 7 months	2/2
YH	5 1 40	1 10 48	8 years 9 months	0/2
YH	17 4 40	13 5 49	9 years 1 month	1/2
S10	28 2 40	13 5 49	9 years 1 month	1/2

* Numerator = number positive, denominator = number inoculated

the frozen state after intervals of 3 years and 9 months to 9 years and 2 months, during the period of the Second World War. Although later attempts to recover frozen pre-war strains have been unsuccessful (Table XXV), this does not imply that the limit of survival times has been approached. Actually, numerous factors may have contributed to the poor recovery rate in 1949. For instance, during some preceding period the

TABLE XXV LOSS OF VIABILITY DURING 1948-49 PRESUMABLY DUE TO FAILURE TO MAINTAIN SUFFICIENTLY LOW TEMPERATURE

Strain	Date frozen	Date thawed	Interval (months)	Result
L W	28 1 49	25 3 49	2	0/2
S6	30 1 48	25 3 49	2	1/2
C J	27 8 46	25 3 49	3	0/2
H W	22 4 48	25 3 49	11	0/2
A G	9 1 48	25 3 49	14	0/2
M J	21 5 48	25 3 49	10	0/2
M S S	12 1 48	25 3 49	14	0 2
F R	10 5 48	25 3 49	10	0 2
L W	24 9 48	16 9 49	12	0 2

dry-ice supply had been allowed to become low, and more frequent use of the box had led to less satisfactory conditions of refrigeration

Later studies, some of which have been reported by Hollander & Nell [20] have attempted to measure the damage which occurs during freezing and thawing, and during storage In some of these studies it was found possible to make preliminary observations on more conveniently cultivatable micro-organisms It is believed that common micro-organisms are suitable models, not only for treponemes, but also for the study of the general laws which govern the freezing and preservation of diverse biologic materials, including spermatozoa, red blood cells, and tissue cells These systems in particular have received a good deal of attention since the remarkable observations of Polge, Smith & Parkes,[42] Smith & Polge [54] and Polge [41] that, with the help of glycerol, spermatozoa can regain their physiological activity after freezing and storage at the temperature of dry ice.

Weiser & Osterud [71] should be credited with calling attention to the basic distinction between the two sorts of damage which occur during the maintenance of bacteria at low temperatures. These two types of damage have been designated freezing damage and storage damage

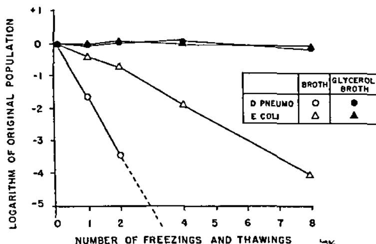
Freezing damage

The measurement of freezing damage in the studies reported by Hollander & Nell [20] was accomplished by the determination of survivors after multiple cycles of freezing and thawing. It had often been noted that repeated freezing was more injurious than a single freezing Turner & Brayton,[65] for example, reported that this was the case with relapsing

fever spirochetes Hollander & Nell [20] found that the survivor curves based upon repeated cycles of freezing and thawing could be used to compare the rates of damage *with different conditions of freezing By this means* it was shown that glycerol protected *Escherichia coli, Diplococcus pneumoniae*, and *T pallidum* from freezing damage.

The survival of *E. coli* and *D pneumoniae*, after repeated freezings and thawings, was determined by plate counts (Table XXVI) The survival curves (Fig 6) constructed from these data indicated that the rate of destruction was lower when glycerol was present in the media.

FIG 6. SURVIVAL OF BACTERIA AFTER SERIAL FREEZING AND THAWING WHILE SUSPENDED IN GLYCEROL



The surviving numbers of Diplococcus pneumoniae *and* Escherichia coli *were measured by plate counts after the indicated number of freezings and thawings The bacteria were suspended in broth or in broth to which 15% glycerol had been added*

Reproduced from Hollander & Nell,[20] *by kind permission of the editors of* Applied Microbiology

The motility of treponemes after freezing and thawing, when various concentrations of glycerol were present in the media, was then examined (Table XXVII) It appeared that motility persisted much longer when 15% glycerol, or more, was present

The virulence of frozen treponemes was next examined in the experiment summarized in Table XXVIII, in order to learn whether the better preservation of motility in the presence of glycerol coincided with better survival Since the incubation period is directly related to the size of the inoculum, the number of living virulent organisms can be estimated from observed

TABLE XXVI EFFECT OF GLYCEROL ON THE PLATE COUNTS OF "ESCHERICHIA COLI" AND "DIPLOCOCCUS PNEUMONIAE" AFTER FREEZING AND THAWING*

Number of times frozen	Broth		Glycerol broth	
	E coli	*D pneumoniae*	*E coli*	*D pneumoniae*
0	750 000	1 500 000	760 000	620 000
1	320 000	35 000	750 000	610 000
2	161 000	590	890 000	680 000
4	980	0	790 000	760 000
8	7	0	550 000	430 000

* From Hollander & Nell[30]

TABLE XXVII EFFECT OF GLYCEROL ON THE MOTILITY OF "T PALLIDUM" AFTER FREEZING AND THAWING*†

Number of times frozen	% Glycerol					
	0	5	10	15	20	25
0	96	—	100	—	—	96
1	36	84	84	100	100	88
2	10	68	92	92	88	92
4	2	56	76	92	88	88
8	—	12	56	84	80	92
16	—	2	32	48	34	22

* Numbers are percentage of organisms showing motility
† From Hollander & Nell[30]

TABLE XXVIII EFFECT OF GLYCEROL IN FROZEN AND THAWED SUSPENSIONS OF "T PALLIDUM" ON THE INCUBATION PERIOD OF SYPHILIS IN RABBITS*

Number of times frozen	Serum saline [a]		Glycerol serum saline [a]	
0	5	7	7	7
1	13	14	7	8
2	26	27	Not inoculated	
4	Negative [b]	Negative [b]	7	7
16	Not inoculated		10	12

* From Hollander & Nell[30]
[a] Numbers are incubation periods in days
[b] Negative animals developed no lesions and the popliteal lymph nodes were not infectious for normal rabbits

incubation periods, and from these data the amount of destruction by freezing can be determined

Organisms suspended in either serum saline or serum saline with 15% glycerol added were subjected to freezing and thawing, and then inoculated into rabbits according to the scheme shown in Table XXVIII. Each rabbit received approximately 500 000 treponemes at each of 8 sites on the shaved surface of the back. The expected incubation period for this size inoculum is about 6 days

The rabbits inoculated with the unfrozen serum saline suspension duly developed lesions after 5 and 7 days When the same emulsion was frozen once, the incubation periods were 13 and 14 days, and when frozen twice 26 and 27 days Since 4 days corresponds to a tenfold decrease in the inoculum, these incubation periods signify that the effective inocula were reduced to the order of 5000 treponemes by one freezing, and to only 5 treponemes by 2 freezings, and indicate that the rate of destruction was at least 99% at each freezing If this rate of destruction were to continue, no virulent organisms would be present after 3 or 4 freezings; in fact, in this experiment rabbits inoculated with material frozen 4 times remained symptomless for 60 days, and their popliteal lymph nodes when transferred to other animals were non-infectious.

In contrast, when glycerol was present in the treponeme suspension, the virulence measured by the incubation period was not altered by a single freezing nor even by as many as 4 freezings (Table XXVIII). After 16 freezings the mean incubation period was prolonged by 4 days, corresponding to a loss of virulence of about 90%, however, it should be noted that this suspension was in the thawed state much longer than the others, and was therefore exposed to atmospheric oxygen for a much longer time.

Storage damage

With regard to storage damage—the second type of damage involved in preservation at low temperatures—, Weiser & Osterud [71] described the deterioration during storage as " a direct function of time and temperature". This principle is well illustrated by our observations on the results of storing treponemes frozen by the glycerol method (Table XXIX) Multiple aliquots of a suspension of treponemes in glycerol were stored in sealed glass ampules at −15°C, −40°C and at −70°C, respectively. After designated intervals the vials were opened and rabbits were inoculated intracutaneously on the back to determine the deterioration by the length of the incubation period. At −15°C the material was inactive after 1 month; at −40°C this material was active at 1 month, but not at 2 months, while at −70°C the treponemes were equally active after 1, 2, and 9 months

On the basis of these experiments (Tables XXVI, XXVII, XXVIII and XXIX) 15% glycerol in 10% inactivated normal serum has been adopted

TABLE XXIX. INFLUENCE OF TIME AND TEMPERATURE ON THE SURVIVAL OF "T PALLIDUM" INFECTIVITY OF SUSPENSIONS OF "T PALLIDUM" AFTER FREEZING IN 15% GLYCEROL AND STORAGE AT VARIOUS TEMPERATURES *†

Temperature and duration of freezing and storage		Incubation periods of individual rabbits in days			
Unfrozen		14	15	16	17
Frozen without storage		14	15	15	17
−15°C	1 month	neg	neg	neg	neg
−40°C	1 month	24	40	neg	neg
−40°C	2 months	neg	neg	neg.	neg
−70°C	1 month	14	14	16	16
70°C	2 months	13	14	16	16
70°C	9 months	15	15	15	16

* Rabbits were inoculated intracutaneously at a number of sites on the back, each site receiving 500 000 treponemes

† From Hollander & Nell [39]

as the routine diluent in the preparation of emulsions which are to be frozen and stored Only 4 of 17 recent consecutive attempts to preserve strains of treponemes in emulsions of hamster lymph nodes by this method (Table XXX) were unsuccessful; and in the 4 failures rabbit serum, which may not have been inactivated, was inadvertently used as the diluent instead of hamster serum saline

Freezing and drying

Turner [60] in 1938 reported little or no success in preserving treponemes after freezing and drying One specimen produced lesions 24 hours after drying, but this was attributed to incomplete dehydration. Turner, Bauer & Kluth [62] later reported failure to preserve material from 9 rabbits infected with *T. pallidum* and 6 with *T pertenue*. Hampp,[17] however, using whole testis for freezing, was able to infect rabbits from each of 6 specimens believed to have been thoroughly dehydrated and stored for periods up to 66 days The use of the whole testis appears to offer good protection against freezing damage, and frozen infected testes for routine immobilization tests have been successfully employed by Chorpenning, Sanders & Kent [7]

Discussion of preservation by freezing

Effective preservation of treponemes at low temperatures requires first of all an emulsion of a type which will keep its vitality for a relatively long

TABLE XXX INFECTIVITY OF TREPONEMES IN EMULSIONS OF HAMSTER LYMPH NODES AFTER FREEZING AT APPROXIMATELY −70°C FOR VARYING PERIODS IN SUSPENDING MEDIUM CONTAINING 15% GLYCEROL

Strain	Frozen	Thawed	Interval (months)	Result
Baghdad A	17 6 53	10 3 54	9	1/2
Baghdad B	22 7 53	10 3 54	8	1/2
Chicago	9 6 53	20 11 53	5	3/3
Mexico A	11 6 53	9 8 54	14	2/2
Nichols	11 6 53	16 8 54	14	2/2
YD post 1949	18 6 53	10 3 54	9	1/1
Cuniculi A	30 6 53	16 8 54	14	0/2
Bosnia A	17 6 53	9 3 54	9	1/1
Bosnia B	17 6 53	9 3 54	9	1/2
Syria A	19 6 53	10 3 54	9	0/2
Syria B	17 6 53	10 3 54	9	2/2
Iraq B	11 6 53	16 8 54	14	0/2
Haiti A	18 6 53	9 3 54	9	2/2
Haiti B	11 6 53	20 11 53	5	2/2
Indonesia B	19 6 53	30 11 53	5	4/4
Indonesia B	9 6 54	16 8 54	5	0/2
Indonesia B	19 6 53	21 9 54	13	2/2

period at ordinary temperatures It is believed that the processes of deterioration which occur at ordinary temperatures are retarded, but probably not arrested completely, at the temperature of dry-ice storage, namely, about −70°C

Secondly, the organisms must be protected from excessive damage during the process of freezing The nature of this damage and its possible causation was discussed by Hollander & Nell,[20] who were of the opinion that the damage was caused by mechanical compression from the expansion involved in the formation of ice Others have preferred different explanations for freezing damage, such as alteration in the electrocyte balance, or the formation of ice crystals Whatever the precise mechanism, it seems certain that the presence of 15% glycerol offers a relatively effective protection against this damage

Thirdly, the storage temperature must be as low as practicable A temperature of −70°C in a dry-ice cabinet has been successful for periods of years Specimens should be stored in sealed glass ampules to protect them from possible damage by carbon dioxide

Cultivation of Treponemes

The cultivation of pathogenic treponemes has been one of the challenges of experimental bacteriology. Schereschewsky [48] in 1909 seems to have been the first to claim successful cultivation. He later [49] purified the culture and infected rabbits. Kast & Kolmer [21] in 1929 listed 29 investigators who had presented similar claims. Later, in 1933, after many failures, Kast & Kolmer [22] reported a single successful culture, but eventually they concluded [23] that the cultivation of pathogenic treponemes remained an unsolved problem Noguchi [39] described, in great detail, the methods by which he had been able to culture strains almost routinely. The strains isolated by Noguchi became avirulent for rabbits within 4 months of culture.[37] Schereschewsky, however, has steadfastly insisted that his cultures are actually pathogenic, and he has recently reported [50] continuing success in cultivation and infection of mice using essentially his original methods

The nature of the cultivated strains and their relationship to the pathogenic strains have been examined by many investigators, including Zinsser, Hopkins & McBurney,[76] and Eagle & Germuth [11] The problem resolves itself into a question of whether these strains are in the nature of mutants which lose their pathogenicity and at the same time become less fastidious in their growth requirements; or whether they are chance contaminants mistakenly identified as *T pallidum* The cultured strains are themselves a heterogeneous group On the basis of agglutination and complement-fixation tests Eagle & Germuth [11] found that the Nichols and Noguchi strains were serologically identical, the Reiter and Kazan strains formed a second group but were not identical, and the Kroo strain was serologically in a group by itself.

The Reiter treponeme

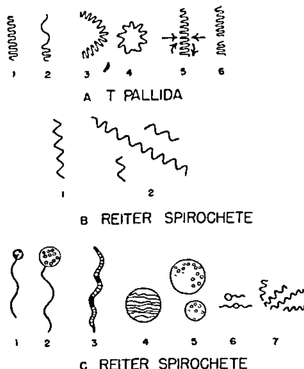
In 1926 Reiter [43] mentioned the isolation of a strain of treponeme According to Eagle & Germuth [11] the details of this isolation were never published This organism, like the other cultured strains, has consistently been non-pathogenic for laboratory animals. It was originally regarded as an avirulent variant of *T. pallidum*, but since numerous studies, which will be examined in Chapters 5 and 7, failed to reveal a close antigenic relationship to pathogenic treponemes, it should perhaps more properly be regarded as belonging to a distinct species of treponeme.

Nevertheless, much work has been done on the Reiter spirochete from the standpoint of cultivation, life cycle, antigenic fractionation and *in vitro* drug testing, in the hope that the data thus obtained would give leads for further study of syphilis and related treponemes. It is of course questionable how far the results of these studies are pertinent to problems concerning the pathogenic varieties of treponemes.

In our laboratory Gelperin [13, 14] has studied the morphological and cultural characteristics of the Reiter spirochete and has carried out certain fractionation procedures and cross-immunity studies With reference to the morphological description of the Reiter treponeme, we quote from Gelperin·

"The organism grows readily in an anaerobic broth or Brewer's thioglycollate medium containing heated serum The various developmental phases in the growth of the organism which have been observed during incubation at 37°C are shown in Fig 1B and C [Fig 7]

FIG 7 MORPHOLOGY OF CULTURED REITER TREPONEMES



Diagrammatic representation (see text for description)

Reproduced from Gelperin,[13] by kind permission of the editors of the American Journal of Syphilis, Gonorrhea and Venereal Diseases

The organism portrayed in B is readily distinguishable from *T pallidum* in that it is 1 5 to 3 times as thick, usually two to four times as long, and with coarse spirals Its movement are languid, the brisk movements characteristic of *T pallidum* are not observed This form is regularly seen if the organism is subcultured every five to seven days in medium

containing from 0.5 to 0.7 per cent agar. When the organisms are incubated for two to three weeks in a medium containing agar, morphologic changes occur (Figure 1 C-1 and 2) Balloon-like, transparent spheres which contain a scattering of small, round, translucent, stationary bodies appeared at one end of some nonmotile spirochetes. Similar structures have been described for other nonpathogenic spirochetes, although their existence has been disputed After four to six weeks of cultivation, a small number of spirochetes distended by rows of these translucent bodies are seen (C-3); free, balloon-like forms (C-4 and C-5) are also seen at this time. Form C-4 appears to be a monolayer of non-motile spirochetes wrapped around the translucent bodies described above. After several months of incubation, the predominant form is the balloon-like structure shown in C-5 " [13]

Gelperin suggested that these balloon-like structures represented an " encystment of the organism which occurs when propagation becomes untenable ". They appeared rapidly when the medium was made less suitable for growth by the omission of agar or agitation; by failure to heat the serum, or by acid conditions in the medium.

When a culture containing the usual culture appearances of 1, 2, and 3, and the balloon-like structures, C-4 and 5, was subcultured into fresh medium,

" the developmental phase pictured in C-6 appears Great numbers of this phase are present individually as well as in agglomerate masses, and Forms C-1, 2, 3 and 5 are no longer present Form C-4 has been observed to shed its layer of spirochetes on subculture, the organisms appear to revive slowly at one end, and as activity progresses throughout their length, the active portion detaches itself completely. Within forty-eight hours, many short, very active spirochetes (C-7) which are almost as thin as *T pallidum* appear, and phase C-6 has largely disappeared the number of organisms (C-7) present , indicates their development from the " cyst " form (C-6) rather than from the relatively few nonmotile spirochetes contained in the initial inoculum The fate of the shells in C-4 and C-5 is unknown. By the fourth or fifth day, the languid, longer, and *thicker spirochetes, pictured in B-1, predominate* " [13]

Gelperin noted that the organisms grew readily in anaerobic broth or thioglycollate media, provided that heated serum was added Steinman and his collaborators [12, 40, 55 58] have published a number of reports on a detailed investigation of the cultural requirements of this organism, including the latest information in the field

In summary, it may be stated that while spirochetes which bear some resemblance morphologically to pathogenic treponemes can readily be grown on artificial media, these organisms are regularly non-pathogenic for the laboratory animals commonly used in the study of the treponematoses, and do not appear to be closely related antigenically to pathogenic treponemes. On the other hand, occasional reports have appeared in the literature to the effect that a pathogenic treponeme has been grown on artificial media Regardless of the validity of those observations, up to the time of writing no method of cultivation has been described which can be repeated successfully by competent bacteriologists.

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containing from 0 5 to 0 7 per cent agar When the organisms are incubated for two to three weeks in a medium containing agar, morphologic changes occur (Figure 1 C-1 and 2) Balloon-like, transparent spheres which contain a scattering of small, round, translucent, stationary bodies appeared at one end of some nonmotile spirochetes. Similar structures have been described for other nonpathogenic spirochetes, although their existence has been disputed After four to six weeks of cultivation, a small number of spirochetes distended by rows of these translucent bodies are seen (C-3), free, balloon-like forms (C-4 and C-5) are also seen at this time Form C-4 appears to be a monolayer of non-motile spirochetes wrapped around the translucent bodies described above After several months of incubation, the predominant form is the balloon-like structure shown in C-5." [13]

Gelperin suggested that these balloon-like structures represented an "encystment of the organism which occurs when propagation becomes untenable" They appeared rapidly when the medium was made less suitable for growth by the omission of agar or agitation; by failure to heat the serum, or by acid conditions in the medium

When a culture containing the usual culture appearances of 1, 2, and 3, and the balloon-like structures, C-4 and 5, was subcultured into fresh medium,

" the developmental phase pictured in C-6 appears Great numbers of this phase are present individually as well as in agglomerate masses, and Forms C-1, 2, 3 and 5 are no longer present Form C-4 has been observed to shed its layer of spirochetes on subculture, the organisms appear to revive slowly at one end, and as activity progresses throughout their length, the active portion detaches itself completely Within forty-eight hours, many short, very active spirochetes (C-7) which are almost as thin as *T pallidum* appear, and phase C-6 has largely disappeared the number of organisms (C-7) present indicates their development from the "cyst" form (C-6) rather than from the relatively few nonmotile spirochetes contained in the initial inoculum The fate of the shells in C-4 and C-5 is unknown By the fourth or fifth day, the languid, longer, and thicker spirochetes, pictured in B-1, predominate " [13]

Gelperin noted that the organisms grew readily in anaerobic broth or thioglycollate media, provided that heated serum was added Steinman and his collaborators [12, 40, 55 58] have published a number of reports on a detailed investigation of the cultural requirements of this organism, including the latest information in the field

In summary, it may be stated that while spirochetes which bear some resemblance morphologically to pathogenic treponemes can readily be grown on artificial media, these organisms are regularly non-pathogenic for the laboratory animals commonly used in the study of the treponematoses, and do not appear to be closely related antigenically to pathogenic treponemes. On the other hand, occasional reports have appeared in the literature to the effect that a pathogenic treponeme has been grown on artificial media. Regardless of the validity of those observations, up to the time of writing no method of cultivation has been described which can be repeated successfully by competent bacteriologists

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Chapiter 5

IMMUNITY PHENOMENA IN THE TREPONEMATOSES

During the great epidemic wave of syphilis that swept over Western Europe in the early years of the 16th century, it was recognized that one attack of the disease induced a degree of immunity to a second attack. About a hundred years ago reinoculation and auto-inoculation experiments in human beings, carried out largely as a method of differentiating the primary lesion of syphilis from chancroid, provided clear evidence that syphilitic infection gives rise to partial immunity and that the degree of resistance, at least in the early stages of the disease, in general bears a direct relationship to the duration of infection [7]

Discovery of the causative agent of syphilis and transmission of the infection to laboratory animals led to illuminating investigations of immunity in this disease, notably by Neisser, Uhlenhuth, Kolle and Chesney and their associates Those studies have been ably reviewed in Chesney's monograph *Immunity in syphilis*,[7] and in his later Harvey Lecture [8]

From the earlier studies came the notion that immunity in syphilis is a unique phenomenon, differing qualitatively from immunity in many other infectious diseases, and that resistance appears to be linked closely to the presence of the infecting agent. Neisser for example promulgated the term "*Infektionsimmunitat*" which carried the implication that cure of the disease led to the disappearance of resistance

It was principally Chesney and his group who demonstrated the nature of immunity in experimental syphilis, showed the importance of the time element in its development, and clearly demonstrated that, in rabbits, immunity once well established persists long after elimination of the infecting organism This basic concept has been confirmed by a number of investigators, including Magnuson and his associates [33, 34] and by research in our own laboratory

While the above findings seemed to indicate that immunity in syphilis was governed by the same general principles that appeared to control the development and maintenance of immunity in most of the other infectious diseases, the immunological uniqueness of syphilis, and by implication

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which persists in the absence of the disease This conception, if fully substantiated, will dispose of the reinoculation test as a criterion of cure of syphilis

" Recent experiments with rabbits show that the acquired refractory state which develops in these animals during the course of syphilitic infection is more effective against a second inoculation with homologous strains than with heterologous strains There is also evidence to show that it is possible to reinfect treated syphilitic rabbits without the development of a characteristic syphilitic lesion at the site of reinoculation. The time at which treatment is begun and the mode of reinoculation appear to be two factors which play a part in bringing about this type of reaction "

These older observations and ideas have recently been confirmed and substantiated in a number of laboratories, including our own, where somewhat more quantitative experimental methods have been employed. Some of these data will be presented here to indicate the kind of information which forms the basis of our knowledge of the evolution of immunity in experimental syphilis

Relationship of Immunity to Duration of Infection

In one study reported by Magnuson & Rosenau[33] 189 rabbits were infected by either the testicular or the cutaneous route, treated with either penicillin or mapharsen at 3, 6, 12, or 24 weeks, and challenged with serial dilutions of treponeme suspensions 6 weeks after treatment The challenge inoculum either produced symptoms without evidence of resistance, or produced a symptomless infection detectable only by lymph-node transfers, or failed to infect the animals, as attested by the absence of local lesions and negative lymph-node transfers The total number of rabbits in each of these categories after 3, 6, 12 and 24 weeks of the original infection without regard to the size of the challenge inoculum are listed in Table XXXI,

Duration of first infection (weeks)	Symptomatic [a]	Asymptomatic [b]	Immune [c]
	31	10	8
6	9	12	14
12	2	26	14
24	1	29	33

* Adapted from Magnuson & Rosenau[33]
[a] Lesions at site of challenge inoculum
[b] No lesions at site of challenge, infection demonstrated by positive lymph-node transfer
[c] No lesions at site of challenge and lymph-node transfers negative

the other treponematoses, largely vanished with the demonstration in our laboratory that antibody, specific for the *Treponema*, not only accompanied infection but appeared to be intimately involved in immune phenomena.

Immunological investigations in this laboratory on the treponematoses have been concerned principally with two broad problems: (*a*) the nature, role and methods of demonstrating specific antibodies to *Treponema*, with which this chapter is concerned; and (*b*) the immunological relationships among species and strains of *Treponema*, which will be treated in Chapter 8 From studies on the first problem have come not only new concepts of the underlying mechanism of immunity in treponemal diseases, but in addition new serological tests (primarily the treponemal immobilization test and the treponemal agglutination test) which can be applied to the study of the naturally occuring disease in man and the experimental disease in animals From studies on the second problem have come data bearing on the biological relationships existing among this group of micro-organisms

Evolution of Immunity in Syphilitic Infection

The most thorough-going studies on immunity in the treponemal infections have been with experimental syphilis. Less extensive studies on yaws and cuniculi infections indicate that these latter follow much the same pattern as syphilis as regards the immune reaction. The evidence concerning the evolution of this immunity was well described by Chesney [7] in 1927:

" With reference to acquired immunity to syphilis, clinical experience has demonstrated that second attacks of this infection in the same individual are extremely rare Inoculations of patients in various stages of the disease with active syphilitic virus have shown that the syphilitic individual gradually acquires a resistance against luetic virus introduced into the skin from without This resistance is not absolute, however, but appears to be more pronounced during the later stages of the disease Inoculation of patients presenting secondary or tertiary manifestations of syphilis with virulent syphilitic virus under certain [illegible] characteristic of the [illegible] ery [illegible] as [illegible] a second infection. In the rabbit this resistance is first manifest about 6 to 8 weeks after inoculation and appears to be firmly established by the fifteenth week of the disease [illegible] for its development There is some [illegible]

TABLE XXXII. RESULTS OF CHALLENGE INOCULATIONS IN RABBITS PREVIOUSLY INFECTED WITH SYPHILIS AND GIVEN CURATIVE THERAPY AT VARIOUS INTERVALS AFTER FIRST INFECTION
BOTH INOCULATIONS INTRACUTANEOUS WITH NICHOLS STRAIN *

Number of treponemes in challenge inoculum	Duration of first infection at time of treatment			
	3 weeks [a]	6 weeks [a]	12 weeks [a]	24 weeks [a]
2	4/11	0/5	1/10	0/11
20	8/11	1/5	1/10	0/11
200	11/11	3/5	1/10	0/14
2000	11/11	3/5	1/10	0/14
20 000	11/11	3/5	2/10	0/14
200 000	11/11	3/5	1/10	0/14
2 000 000				0/3
10 000 000				0/3

* Adapted from Magnuson & Rosenau [illegible]

[a] Numerator = number of animals developing a lesion at the site of indicated challenge dose; denominator = number of animals challenged

TA [illegible]

Group	Treatment begun	Challenge inoculation				Control rabbits	
		Interval *after* treatment	Challenge dose	Number of rabbits with lesions	Mean incubation period (days)	Number *with* lesions	Mean incubation period (days)
A	3 weeks	2 weeks	500	4/6	20 [a]	3/3	19
B_1	2 months	2 weeks	500 50 000 5 000 000	3/5 2/5 5/5	21 [a] 21 [a] 6	3/3 3/3 3/3	17 10 6
B_2	2 months	1 year	500	9/9 [b]	15 [a]	5/5	18
C	6 months	2 weeks	500 50 000 5 000 000	0/4 [c] 0/5 [c] 0/4 [c]	— — —	2/2 3/3 3/3	19 10 5

[a] Lesions much smaller than those of controls
[b] Lymph-node transfer positive in 8 animals, negative in one
[c] Lymph-node transfer negative in all animals

It is apparent that, with continuing infection, immunity develops first to a stage where infection is symptomless, and later to the point where no infection is established in a proportion of the animals.

It is evident from Table XXXI, however, that while the foregoing summary is a fair representation of the picture as a whole there is marked variation from rabbit to rabbit: even after 3 weeks a small proportion of animals had sufficient immunity to prevent infection, while after 6 months only about half the animals were completely immune, and an occasional animal even developed *symptomatic infection*

In another series of inoculations Magnuson & Rosenau [33] infected a group of rabbits with the Nichols strain, administered curative amounts of penicillin after 3, 6, 12, or 24 weeks, and then challenged them with serial dilutions of homologous treponemes In this experiment both the initial and the challenge inoculations were intracutaneous.

The results as shown in Table XXXII indicate that after 6 weeks of infection there had developed a significant degree of immunity, while after 12 weeks most of the animals, and after 24 weeks all of the animals, were refractory to challenge inoculation

Data somewhat similar to the foregoing have been accumulated in our own laboratory as a result of work undertaken in collaboration with Dr Robert A. Nelson, *jr., on the relation of immobilizing antibody to resistance* [62]

Sixty rabbits were infected by intratesticular inoculation of the Nichols strain of *T pallidum*; all developed typical syphilitic orchitis. Curative treatment consisting of a total dose of 32 mg of crystalline penicillin G administered in aqueous solution twice daily for 8 days was given to groups of animals at different intervals after initial infection At varying intervals *after the initiation of treatment, challenge inoculation with a measured* inoculum was made intracutaneously at 4 sites on the rabbit's back Groups of normal rabbits were similarly inoculated as controls

The results of these experiments are shown in Table XXXIII Even after as short an interval as 3 weeks after the initial inoculation (Group A), demonstrably increased resistance was present, some animals showed no lesions, while in others the lesions failed to progress to the same size as those in normal rabbits similarly inoculated In this experiment the incubation period for the first infection was about 10 days in all animals, so that clinically recognizable syphilitic lesions had been present in Group A for about 10 days at the time treatment was instituted.

The immunity developed by Group B1 animals, which were treated at 2 months, seemed to be of about the same order of magnitude as in Group A, although those animals challenged with 500 and 50 000 treponemes at each site showed a significantly longer incubation period as well as smaller lesions than the respective control groups When a large inoculum was given, however, no difference in the incubation period was noted, although the resulting lesions remained smaller than those in the controls.

TABLE XXXIV RESULT OF CHALLENGE INOCULATION WITH A HOMOLOGOUS SYPHILIS STRAIN IN RABBITS TREATED 28 DAYS AFTER INTRATESTICULAR INOCULATION WITH VARYING NUMBERS OF TREPONEMES

Group	Number of rabbits	First infection		Median pre-challenge TPI titer	Intradermal challenge inoculation 500 treponemes X 4	
		Number of treponemes inoculated	Mean incubation period (days)		Mean incubation period (days)	Maximum size of lesion
Controls	6	0	—	0	17 1	++++
A	5	500	25	5	15 6	++++
B	6	50 000	16	32	18 1	++
C	3	25 000 000	8	110	18 0	+

should be noted that Group A animals showed a shorter incubation period than did the animals of the other groups, including the controls

Additional evidence showing that the mere presence of relatively small numbers of treponemes does not lead to immunity, except after many weeks, is provided by the experiments of Hollander, Turner & Nell [22] in which subclinical infection was maintained over a period of months by the judicious administration of penicillin Even after 20 weeks, the subsequent evolution of the syphilitic disease process upon the withdrawal of penicillin was essentially that which might have been expected from fresh infection During this long period of subclinical infection, rabbits did not develop Wassermann antibodies or treponemal immobilizing antibodies This experiment will be described in detail in Chapter 8 Magnuson, Thompson & Rosenau [34] in a somewhat similar type of experiment likewise reached the conclusion that while some degree of immunity develops during the prolonged period of asymptomatic infection this is of a much lower order of magnitude than that developing during symptomatic infection of the same duration

Influence of Size of Challenge Inoculum

Many experiments designed to test the degree of immunity in syphilis have been devised on the premise that the size of the challenge inoculum is an important factor in determining whether or not the experimental animal becomes infected Close inspection of pertinent experimental protocols, however, reveals evidence which indicates that host resistance operates largely independently of the size of the challenge inoculum

On the other hand, the animals of Group B2, which were treated at the same time-interval as those in Group B1, seemed to have lost some degree of resistance 1 year after the institution of curative treatment. In the former group all animals showed lesions, but these did not reach nearly the same size as those in the controls.

It should be noted that the mean incubation period in the previously infected animals of this group (B2) was significantly shorter than in the controls Since the incubation period in individual controls was quite uniform, it is possible that this result reflects an acceleration of the division time of treponemes in the partially immunized group. It has been noted that at certain critical concentrations a number of antibiotics seems to cause increased growth of such organisms as *Staphylococcus aureus* The question might be raised as to whether antibodies in just the right concentration may also lead to stimulation of growth, instead of to inhibition.

Group C animals, in which the infection was allowed to progress untreated for 6 months, were solidly immune when challenged with either *small or large numbers of treponemes shortly after treatment*

Relationship of Antigenic Mass to Immunity

An experiment similar to the foregoing was carried out in our own laboratory by Turner & Nelson [62] to determine the effect of certain quantitative relationships on the development of immunity. Three groups of 6 rabbits each were inoculated in both testes with approximately 500, 50 000, and 25 000 000 treponemes of the Nichols strain, respectively The incubation period of the resulting orchitis varied, as expected, with the size of the inoculum

On the 28th day after original inoculation curative treatment with crystalline penicillin G in aqueous solution was begun One week after the last injection of penicillin all surviving animals, together with 6 normal controls, were inoculated intracutaneously with 500 treponemes at each of 4 sites on the animals' back. The results are shown in Table XXXIV.

This experiment indicates that the development of resistance is not dependent alone on the duration of infection, but is also influenced by the degree of antigenic stimulus stemming from the multiplication of treponemes in the host Since the time period was the same in the 3 groups, the degree of immunity seems to have been related to the stage of clinical evolution of the lesions

In this experiment, for example, all 3 groups of animals had been infected for the same length of time, yet Group A, which had detectable lesions for only 3 days at the time treatment was instituted, had little if any enhanced resistance, while Group C, which had had extensive lesions for 20 days, showed a high degree of immunity to challenge inoculation It

All the animals were given curative amounts of penicillin 7-10 months after the first inoculation, and 6 weeks later were challenged intradermally with tenfold dilutions containing from 100 to 1 000 000 treponemes of the same strain Rabbits failing to develop lesions on challenge inoculation were subjected to lymph-node transfer.

TABLE XXXVI RESULTS OF INFECTIVITY TESTS FOLLOWING CHALLENGE INOCULATION OF RABBITS GIVEN CURATIVE PENICILLIN TREATMENT AFTER 3 MONTHS FIRST INFECTION, INTRATESTICULAR, CHALLENGE INOCULATION, INTRADERMAL (BOTH WITH NICHOLS STRAIN) † *

Number of treponemes in challenge inoculum	Results of infectivity test [a]
100	1/4
1 000	2 5
10 000	3 4
100 000	1 4
1 000 000	1 5

* None of the animals developed lesions

† Adapted from McLeod & Magnuson [35]

[a] Numerator = number of rabbits with infective lymph nodes , denominator = number of animals challenged with indicated dose

The results are shown in Table XXXVI Again it is evident that reinfection occurs largely independently of the size of the challenge inoculum Additional supporting evidence for this statement can be found in another paper by Magnuson, Thompson & Rosenau [36]

Persistence of Immunity after Curative Treatment

The validity of the notion of Chesney [7, 8] that once immunity has developed it persists, despite the eradication of the infecting organisms, is illustrated by the data of Magnuson, Rosenau & Clark [34] summarized in Table XXXV A large group of rabbits were infected with the Nichols strain, given curative treatment with penicillin 3 months after infection, and then challenged either 1½, 2, 3, 6, or 12 months after elimination of the first infection In this experiment the initial inoculation and the challenge inoculation were given into the same testis

The results, as shown in Table XXXV, indicate that no appreciable change in the immune status after curative treatment took place with the lapse of time as determined by challenge inoculation. In other words, immunity once established persisted for at least one year after the infection had been eliminated Although none of the 110 rabbits developed a clinically recognizable lesion on challenge inoculation, about two-thirds of

For example, analysis of the results reported by Magnuson & Rosenau [33] shows that some animals developed no lesions when inoculated with tenfold serial dilutions of a suspension of treponemes containing from 2 to 200 000 organisms, while other animals developed lesions at all sites of inoculation regardless of whether the number of treponemes inoculated was 2 or 200 000 (See Table XXXII, page 127.)

Likewise, in another experiment reported by Magnuson, Rosenau & Clark [34], which is summarized in Table XXXV, the proportion of rabbits developing a second infection did not vary significantly either with the lapse of time after curative treatment, or with the size of the challenge dose; of 27 animals receiving as small a challenge dose as 200 treponemes, 9 became infected These data support the conclusions drawn from the previous experiment to the effect that reinfection in syphilis is primarily determined by the immune status of the host, and is not materially influenced by the dose of challenge inoculum

It is also of interest that in many animals in the foregoing experiment symptomless infection was accompanied by a sharp rise in Wassermann antibody. Unfortunately, no means were available of testing for immobilizing antibody at the time these experiments were performed.

In a more recent experiment in which the primary purpose was to study cross-immunity between syphilis and yaws, McLeod & Magnuson [30] infected 23 rabbits with the Nichols strain by intratesticular inoculation.

TABLE XXXV RESULTS OF INFECTIVITY TESTS ON LYMPH NODES OF RABBITS INOCULATED INTRATESTICULARLY WITH NICHOLS STRAIN, GIVEN CURATIVE THERAPY AFTER 3 MONTHS, AND CHALLENGED INTRATESTICULARLY AFTER VARIOUS INTERVALS *†

Number of treponemes in challenge inoculation	Time of challenge after curative therapy			
	6 weeks [a]	12 weeks [a]	24 weeks [a]	48 weeks [a]
200	2/6	4/10	* 7	2/4
2000	3/5	4/4	4/5	1/3
20 000	6/8	11/11	3/6	4/5
200 000	5/7			
2 000 000		7/10	7/8	3/5
10 000 000	4/6			
Total	20/32	26/35	15/26	10/17

* None of the rabbits developed symptomatic reinfection
† Adapted from Magnuson, Rosenau & Clark [34]
[a] Numerator = number of rabbits with infective lymph nodes, denominator = number of rabbits challenged with indicated dose

Evidently treponemes were by some mechanism held *in situ*, though they were still viable, for a period of several days

Somewhat similar results were obtained by Waring & Fleming[65] who showed that in partially immune rabbits the lymph nodes may remain non-infectious even though local lesions in which motile treponemes can be demonstrated may occur following challenge inoculation. It seems reasonable to assume that specific antibody may have been playing the key role in this situation

Evolution of Immunity in Other Treponemal Infections

Studies somewhat similar to those just described have been carried out in our laboratory on rabbits infected with yaws and cuniculi treponemes. While these studies have been less extensive and incidental to cross-immunity experiments, they indicate that immunity in yaws and cuniculi infection in rabbits follows much the same general pattern as in syphilis (See Chapters 7 and 8.) On the whole, the immune reaction seems to develop somewhat more slowly and is frequently of a somewhat lower order of magnitude than that commonly developing as a result of syphilitic infection

Matsumoto[38] and his associates have also presented numerous experiments substantiating these conclusions McLeod & Magnuson[30] likewise have shown that yaws infection in rabbits gives rise to a high degree of resistance to challenge inoculation with the same strain, of 24 such animals treated 7-9 months after the first infection with our strain YC, none developed lesions upon intracutaneous challenge inoculation of the same strain, and infective lymph nodes were demonstrated in only one animal Most investigators, including McLeod & Magnuson,[27, 30] however, question the reliability of lymph-node transfer as an index of non-infectivity in yaws, since a fair proportion of known but untreated yaws cases in rabbits yield negative results on lymph-node transfer (See Chapter 2.)

Attempted Induction of Immunity with Killed Treponemes

Numerous attempts have been made over the years to induce immunity in laboratory animals with killed treponemes, both the pathogenic and the non-virulent cultivatable variety. In most instances the results have been disappointing

With increasing knowledge concerning immunization procedures in general and immunity in syphilis in particular, factors such as a prolonged period of immunization, more sensitive challenge procedures, and the use of adjuvants, have been embodied in experiments directed to this problem

Thus Eagle & Fleischman[16] injected rabbits intradermally, intramuscularly, intraperitoneally and intravenously with large doses of killed pathogenic *T pallidum* over periods of up to 4 months. Treponemes were killed

the animals acquired a symptomless infection, as indicated by the infectivity of their lymph nodes The proportion of symptomless infections was essentially the same, irrespective of the interval following treatment.

Similar studies showing the persistence of immunity after penicillin therapy have been reported by Arnold and his co-workers.[1 4] Indeed all these studies serve to confirm the basic observations made by Chesney and others in the second and third decades of this century.[7]

The Nature of Latent Syphilis

It is quite clear that rabbits (and human beings) acquire a degree of immunity during the course of syphilitic infection It is equally clear that this immunity is by no means complete In respect of the treponemes introduced during the initial infection, the animal develops the capability of holding them in check to the extent that clinically manifest lesions rarely occur after the 3rd month, except in the eye, which is known to participate less actively in the immune process than most of the other parts of the animal body[66] But virtually without exception the animal is incapable of eliminating the infection; lymph nodes are regularly found to be infectious, and Frazier, Bensel & Keuper[17] have shown that intermittent spirochetemia occurs in some rabbits for at least 3 years after infection.

Rabbits in this stage of infection likewise have limited capabilities of dealing with treponemes introduced from without. The studies cited above demonstrate that, after the first infection was eliminated by treatment, in some animals a new infection was established, but this, however, virtually always remained latent, while in others no infection was established

While we can with justification regard this phenomenon as a common expression of immunity, there is relatively meager information on the precise mechanism involved. How is it that treponemes can remain viable, fully virulent for other animals, and presumably multiply, and yet induce virtually no tissue reaction in the host? Is there a continued interplay between host antibody and the treponeme, a waxing and waning of first one then the other? Or is the treponemal population maintained at a fairly constant level? We have little or no data bearing on these questions

Pertinent to this discussion, perhaps, are the observations reported by Reynolds[53] in which pieces of rabbit syphiloma were implanted under the skin of the lower hind legs of normal and immune animals Observations were made on the fate of the implanted treponemes through darkfield and infectivity tests of the implants and of the popliteal lymph nodes draining the area. In the normal animals motile treponemes increased in the transplants, and the regional lymph nodes were infective 48 hours after implantation. In the immune animals motile treponemes could be observed in the transplants and infectivity demonstrated for as long as 4 days after introduction, but the regional lymph nodes were never demonstrated to be infective.

animal being approximately 1200 million organisms At the end of the injection period the lymph nodes of the animals were non-infective; the blood serum contained no demonstrable immobilizing antibodies. Intradermal challenge inoculation of 200 treponemes (homologous strain) at each of 3 sites was made 2 weeks after completion of the immunization schedule. Ten control animals were similarly inoculated

The lesions resulting from challenge inoculation were significantly smaller in the test animals than in the controls, and in 3 of 4 animals tested the popliteal nodes were non-infective, despite the presence of fairly definite lesions in 2

It should be noted, however, that the test animals in this experiment were in poor general condition as a result of the weekly injections. Since it is known that treponemal infection is often partially suppressed in the presence of intercurrent disease, caution must be exercised in interpreting these results as a manifestation of acquired specific immunity.

Tani, Inoue & Asano[59] have also reported evidence of artificially induced immunity with killed syphilis treponemes Using a vaccine prepared from very large numbers of infected rabbit testes (170 in one experiment) from 3000 million to 8000 million treponemes inactivated by antiformin were injected into each animal over a period of 6 weeks Among a total of 24 immunized rabbits in 3 experiments, 10 developed no lesions on challenge inoculation, 5 showed a modified reaction, and 9 behaved like the control animals Tani and his associates believed that the active immunizing component of the treponeme is deep within the body of the organism and must be " unmasked " by destroying a surface component before the material is immunogenic

Schobl[57] and Schöbl, Tanabe & Miyao[58] reported in 1930 the induction of significant degrees of immunity to yaws infection in Philippine monkeys with a vaccine made of heat-killed yaws treponemes These results, made with relatively few animals, have not been confirmed in another laboratory

Gelperin[19] failed to obtain definite evidence of immunity to challenge with small numbers of syphilis treponemes in rabbits injected with large doses of the Reiter spirochete in an adjuvant mixture (See Chapter 8 for details)

In summary, it can be stated that many investigators over the years have failed to obtain clear-cut evidence of the artificial induction of immunity in the treponematoses with killed organisms, although some results, such as those of Tani, Inoue & Asano,[59] Waring & Fleming,[64] and Schöbl and his associates[57, 58] have been highly suggestive It should be noted that many of the reported experiments have not been made with relatively " purified " and concentrated treponemal emulsions prepared according to recently developed techniques Such experiments might well provide a more solid basis for the suggestive results obtained by some of the investigators referred to above

either with heat, merthiolate or by maintenance in the frozen state at temperatures too high to permit retention of viability. Some animals received as high a total dose as 38 000 million organisms, and in some series the treponemes were suspended in a water and oil adjuvant or mixed with killed tubercle bacilli

Although animals regularly developed Wassermann antibodies, no clear evidence of increased resistance was obtained on intradermal challenge inoculation It is interesting, however, that Eagle & Fleischman [16] noted in some " immunized " animals a shorter incubation period than in the controls, and in one experiment 3 of 5 " immunized " rabbits failed to develop a lesion on testicular challenge with very small doses of treponemes

Magnuson, Halbert & Rosenau [32] likewise failed to obtain evidence of immunity in rabbits following injections with killed treponemes over periods of up to 10 weeks. Rabbit testicular syphilomas served as a source of the Nichols strain of *T pallidum*, and the treponemes were killed by drying *in vacuo*. Injections were made subcutaneously or intramuscularly at weekly intervals in doses of 100 000 000 treponemes; in some instances the treponeme emulsion was combined with an adjuvant consisting of Falba oil and dried *Mycobacterium phlei*. Upon challenge inoculation of 2 to 200 000 treponemes of the homologous strain, none of 18 animals injected with lyophilized treponemes without adjuvant and none of 19 rabbits injected with lyophilized material plus adjuvant showed evidence of enhanced resistance It is of interest that the latter group of animals gave positive results when subjected to standard serological tests, while those of the former group did not

Waring & Fleming,[64] using lyophilized rabbit syphilomas (Nichols strain), injected rabbits intradermally with 0 1 ml of reconstituted material on three successive days in each week Two 4-week courses were administered, separated by a 4-week rest period It was estimated that each animal received a total of approximately 12 000 000 treponemes in each of 3 sites. Six weeks after the last " immunizing " injection the popliteal nodes of all animals were non-infective.

Seven weeks after the " immunizing " schedule was completed the rabbits were challenged by the intradermal inoculation of 200 treponemes (Nichols strain) in each of the 3 previously injected areas, and at 3 other sites Of 9 rabbits so challenged the incubation period and the size of the lesions did not differ significantly from that observed in 20 control animals It was noted, however, that treponemes were more abundant in the lesions of the so-called immunized animals than in those of the control animals.

In another series of experiments by Waring & Fleming,[64] animals were injected intramuscularly with lyophilized extracts of syphilomas emulsified in Falba mineral oil and dried *M. butyricum* Five animals were given weekly injections for 26-27 weeks, the total dose of treponemes for each

secondary syphilis, 135 with previously untreated latent syphilis, 31 with treated latent syphilis, 2 with tertiary syphilis, and 107 presumably non-syphilitic persons Each serum-spirochete mixture was injected intradermally at 4 sites on the rabbits' back, 4 different mixtures (including 1 or 2 control mixtures) being commonly tested in each of 4 rabbits As a group, sera from patients with secondary, latent or tertiary syphilis exerted an inhibitory effect on the development of syphilitic lesions, in comparison with sera from non-syphilitic persons. This effect was manifested by complete suppression of lesions at the sites of inoculation of serum-treponeme mixtures, or by prolongation of the incubation period and smaller lesions

Because of the expense involved in tests of this sort, and because of the lack of precision with respect to any given serum, the method did not represent a practical tool for the study of immunity in man or in experimental animals

Many of the observed features of the method which were obscure at the time can be explained in the light of subsequently acquired knowledge It is now known for example that treponemes survive better at 35°C than at 37°C, the incubation temperature employed in the so-called protection test. (See Chapter 3.) It is also now known that treponemes must be in contact with immune serum for 4-6 hours before immobilization begins to be manifest [45] The incubation period of 6 hours used in the protection test was, therefore, on the borderline of effectiveness and explains in part, perhaps, the absence of clear-cut differences in motility of treponemes in the two kinds of mixtures Similarly, it appeared that the presence of complement enhanced the suppressive action of serum from immune animals, and it is now known that complement is an essential component of the immobilization reaction.[45] In retrospect, it seems likely that the suppression of lesions at the sites of injection of immune-serum spirochete mixtures, or the prolonged incubation period observed, was a direct reflection of the reduced numbers of viable treponemes remaining in the mixtures (See Chapter 2)

An important weakness in the protection test as described above was the fact that under ordinary circumstances the motility, and presumably also the viability, of *T pallidum* and related treponemes became greatly reduced after 4-6 hours, even under the most favorable conditions then available

A number of devices were tried, therefore, in the hope of bringing the treponeme into contact with tissue fluids under circumstances in which the treponemes would have a favourable environment for survival and which at the same time would permit visualization of the organisms by darkfield examination

One method attempted was the production of blister fluid in the rabbit, but satisfactory exudation of fluid could not be obtained Another method on which considerable work was done, but to no avail, was the preparation of skin " pockets " into which tissue fluids might exude and into which

It should be noted in passing that both Magnuson, Halbert & Rosenau [32] and Waring & Fleming [64] injected enormous doses of lyophilized treponemes without producing infection, a result demonstrated by non-infectivity of lymph nodes.

Humoral Expression of Infection and Immunity in the Treponematoses

The serology of syphilis and related diseases is both an old and a new scientific problem; old in the sense that the discovery of the phenomenon of complement fixation by Bordet and Gengou in 1903, and the application of this principle to the diagnosis of syphilis by Wassermann, Neisser and Bruck in 1905, may be counted among the great advances in medicine; new in the sense that the demonstration of antibodies specific for the *Treponema* provides an opportunity for a re-evaluation of the role and *limitations of the standard serological tests employing lipoid antigen in medical practice, and in the understanding of the biology of treponemal* infections.

Since our laboratory has made a significant contribution to these newer developments, it may be appropriate at the present time to attempt a review and an evaluation of several years' work in this field

"*Protective*" *antibodies*

In point of time our first efforts directed toward the demonstration of specific humoral antibodies in the treponemal diseases were made with a combined *in vitro* and *in vivo* system, which we referred to as a protection test. The initial paper on this subject was published by Turner in 1939,[60] *another paper from this laboratory appeared after the Second World War* [63] Since these studies are now mainly of historical interest the details of the experiments will not be presented Briefly, the tests were carried out as described below

An emulsion containing virulent *T. pallidum* was added to serum from either normal rabbits or from untreated immune syphilitic rabbits that had been infected with a homologous strain of *T. pallidum*, the mixture was incubated for 6 hours at 37°C, and injected intracutaneously into normal rabbits Typical syphilitic lesions commonly developed at the sites of inoculation of the normal serum-treponeme mixtures, while at the sites of inoculation of immune serum-treponeme mixtures usually no lesions developed, or else the resulting lesions had a longer incubation period and remained smaller than those produced by normal serum-treponeme mixtures [60]

Subsequent experiments by Turner et al.,[63] using serum from syphilitic and non-syphilitic human beings, gave similar results. Included among the specimens tested were sera from 13 patients with primary syphilis, 35 with

originally described by Nelson & Mayer.[45] In its basic outlines the test is performed as follows Treponemes are extracted from an induced lesion in the rabbit's testis, and this material, together with suitable amounts of the serum to be tested and additional guinea-pig serum as a source of complement, is placed in a maintenance medium under specially designed anaerobic conditions; after overnight incubation of the mixture at 35°C, the test is read by comparing under the darkfield microscope the proportion of motile treponemes in a tube containing the test serum plus complement with the proportion of motile organisms in a tube containing the same serum without complement Usually about 90% of the treponemes in the control tube are motile, while the proportion in the tube with the test serum depends on the presence or absence of specific antibody

The use of syphilis, yaws, bejel and cuniculi treponemes in the TPI test will be described in Chapter 8 An essential requirement for successful tests is that the strain of treponeme utilized be capable of invoking well-developed testicular lesions in the rabbit within an incubation period not exceeding 12 days In the TPI test as ordinarily performed the Nichols strain of *T pallidum* is used routinely

A testicular suspension containing 50-100 million treponemes per ml is prepared in 15% glycerol and frozen at −70°C, to be used as needed Commonly, such material when inoculated in amounts of 0 5-1 0 ml will give rise to a detectable orchitis within 6-8 days, the lesion being ready to use as a source of treponemes in the immobilization test at this time Rabbits infected with strains of treponemes which usually have a longer incubation period are given daily intramuscular injections of cortisone at the rate of 6 0 mg/kg body-weight from the third day after inoculation until treponemes are harvested One animal provides sufficient material for one "run" of the TPI test Chorpenning, Sanders & Kent[9] have reported the use of whole or sliced testes, maintained at −70°C for days or weeks, as a source of treponemes for the TPI test, but from the little experience which we have had with this method, it appears to be inferior to the method described above

The primary objective of the extraction is to obtain a clean suspension of treponemes, free from specific antibody Testes are excised and the material handled under strictly aseptic conditions, since bacterial contamination of the serum-treponeme mixtures may be a cause of unsatisfactory tests The testes are cut into longitudinal and transverse slices with sharp scissors, washed with 10 ml of chilled 0 85% NaCl to remove damaged tissue cells, and placed in 100-ml flasks containing 40 ml of medium

The air in the flask is replaced with a mixture of 5% CO_2 and 95% N_2 by successive evacuation and refilling operations Flask and contents are then agitated for a 2-hour period in a rotary shaker at 35°C After this period a sufficient number of organisms has been extracted from the testicular tissue into the suspending medium The medium containing

treponemes could be placed and removed at intervals *A large number of* such pockets were prepared by our associate, Dr A. Gelperin. A good exudate accumulated within 3 hours, but treponemes introduced into the pocket could not be recovered after about 4 hours. Since the exudate contained many leukocytes as well as tissue fluid, these cellular elements may have played a role in the destruction of treponemes.

Another method explored was the introduction of a treponeme suspension, together with serum from normal or immune rabbits, into the pleural cavity of mice, the hope again being that the survival of treponemes would be sufficient to bring out differences between the normal and immune serum.

Because the aspiration of treponeme-containing fluid from the mouse's *pleural cavity presented difficulties, mice were sacrificed at intervals and the* pleural fluid examined directly. It was found the *T. pallidum* could be recovered up to 9 hours after introduction into the pleural cavity of mice, but not at 12, 18 and 24 hours After 6-9 hours treponeme counts were only 10%-15% as high as those made immediately after injection With a 6-hour period, significant differences were noted between suspensions of treponemes in normal and immune rabbit serum, but the method was not regarded as sufficiently precise to give reliable data on any single serum The high body temperature of the mouse may also have had an adverse effect on the introduced treponemes.

Immobilizing antibodies the treponemal immobilization test

While the studies referred to above were in progress, our associate, Dr Robert A Nelson, jr., was studying factors that affect the survival of treponemes *in vitro*. The results of his studies in this field have been described in Chapter 4, as well as in previously published papers [43, 44, 45] Briefly, an *in vitro* technique was developed whereby *T. pallidum* and related treponemes could be maintained in a viable and motile state for a period of several days

Immediately, this provided a practical and precise method for the detection of specific antibody to these organisms, and resulted in the signal contribution of the treponemal immobilization test by our associates Nelson & Mayer.[45]

It is not the purpose of this monograph to provide a technical source book for the various serological tests employed in dealing with the trepone- [illegible] *World Health Organization* [illegible] It will be [illegible] cerning the pathogenesis of the treponematoses which has been revealed by these new tests, and to this end some pertinent details concerning the tests will also be presented.

Technique of the TPI test With the exception of a few changes noted below, the TPI test is now performed in our laboratory essentially as

A number of technical improvements in the treponemal immobilization test have been suggested by Portnoy, Harris & Olansky,[49] Harris et al,[21] Saurino,[55] and Durel, Borel & Sausse[14] For more complete evaluation of these technical features the reader should consult the issue of the *Bulletin* to which reference has already been made.[5]

While it is possible to obtain quantitative determinations of immobilizing antibody in any particular serum, in actual practice few quantitative studies have been made, largely because of technical problems inherent in the test and the difficulty of obtaining reproducible titers (See Chapter 8)

Agglutinating antibodies: the treponemal agglutination test

It has been known for many years that *T. pallidum* under proper conditions will agglutinate in the presence of syphilitic serum Unfortunately, in the hands of most investigators, organisms either tended to agglutinate spontaneously, or were agglutinated by sera from persons in whom treponemal infection could be reliably excluded. This earlier work has been reviewed elsewhere[20, 28]

Because of the technical difficulties inherent in the treponemal immobilization test it was decided to reinvestigate the phenomenon of agglutination of treponemes, and this task was undertaken in our laboratory by Hardy & Nell[20] A somewhat similar line of investigation was being pursued at the same time by McLeod & Magnuson[28, 31]

Advances in knowledge in other fields helped to throw new light on some of the difficulties previously encountered In the first place, the investigators in this laboratory quickly found that the *in vivo* sensitization of treponemes was a major cause of spontaneous agglutination of treponemes (See the discussion of this subject in Chapter 4)

When suspensions of treponemes are prepared for immobilization tests the use of large inocula in the source animals and early harvesting of the treponemes can eliminate much of the sensitization However, in order to produce the concentrated suspensions required for agglutination new techniques were needed The demonstration of the effect of cortisone[61] provided a method for obtaining an increased number of treponemes, and the methods of extraction[45] devised during the development of the treponemal immobilization test provided a basis for the preparation of agglutinating antigens By combining the use of cortisone with exsanguination, the addition of citrate solution, and centrifugation in the cold, Hardy & Nell[20] have succeeded in obtaining concentrated suspensions of treponemes apparently free from sensitization.

Another troublesome source of sensitization leading to spontaneous agglutination was the occurrence of previous *T cuniculi* infection in some rabbits from which antigen suspensions were prepared This prior contact with the related antigenic material appears to cause an accelerated formation

treponemes is decanted and centrifuged at 1500 r.p.m. for 5 minutes to remove gross tissue particles and spermatozoa. The clear supernate usually contains 1-5 million treponemes per ml. A second extraction of this same tissue may be made in a similar manner, usually the yield of treponemes is about the same as in the initial extraction.

0.4 ml of the treponeme suspension and 0.05 ml of the serum to be tested is pipetted into two test tubes, 25 mm × 100 mm. To one tube is then added 0.05 ml of inactivated guinea-pig serum and to the other an equal amount of fresh guinea-pig serum. These tubes, together with control tubes consisting of one tube in which 0.05 ml of ultrafiltrate of ox serum is substituted for the test serum and a series of tubes with serial dilutions of a standard syphilitic rabbit serum pool, are placed in a Brewer anaerobic jar filled with a mixture of 5% CO_2 and 95% N_2, and are incubated at 35°C for 16-18 hours. At the end of this period the proportion of motile treponemes in each tube is determined by darkfield examination. The proportion of motile treponemes is compared, with and without complement, for each serum tested. The control tubes containing the ultrafiltrate of ox serum and the known negative sera must meet certain standards. All tubes containing complement, in which immobilization of treponemes does not occur, are tested for the presence of residual complement. Data on certain variables affecting the TPI test, together with additional technical details, have been reported by Nelson & Diesendruck [41]

Evaluation of the TPI test. Despite the real scientific merit of the TPI test, both its experimental and clinical usefulness have been limited because of formidable technical difficulties. Among the principal difficulties encountered have been the following:

1. Partial suppression of the infection in rabbits serving as sources of treponemes for the test, this in turn leads either to an inadequate number of treponemes or to *in vivo* sensitization of treponemes with pre-formed antibody. Among the factors contributing to this suppression are (*a*) utilization of a strain of treponemes which is not highly pathogenic for the rabbit; (*b*) environmental animal-room temperatures above 18°C; (*c*) antibiotics inadvertently incorporated in the animal food (see Chapter 6); (*d*) intercurrent or previous infection of source animals with naturally occurring cuniculi infection.

2. Factors leading to poor survival of treponemes *in vitro*. This is a frequent cause of unsatisfactory tests and often the precise difficulty cannot be identified. Among the factors believed at times to be responsible are chemically unclean glassware, toxic elements in the test serum, and inadequate anaerobiosis, oxygen concentration of more than 0.04% in the gas mixture appears to be toxic [14]

3. Deterioration of complement during the test period.

fibrin clots that otherwise frequently occur and enmesh the treponemes when other extraction media are employed; and immediate heating of the resuspended treponemes appears to prevent deterioration during storage.

Satisfactory treponeme suspensions have been prepared with the Nichols and Chicago strains of *T. pallidum*, the Haiti B strain of *T. pertenue*, the Iraq B strain of bejel treponemes, and the cuniculi A strain of *T cuniculi*; presumably any strain sufficiently virulent to produce a testicular lesion rich in treponemes within a period of 12 days will yield a satisfactory antigen.

Agglutination tests are carried out in 10 mm×75 mm test tubes in a total test volume of 0 2 ml, comprising 0 1 ml of antigen and 0 1 ml of diluted serum. Sera are diluted in saline containing 0 005 M ethylenediamine tetracetate (EDTA), adjusted to pH 7 6

For routine diagnostic purposes a 1: 10 dilution of serum is employed to avoid a slight prozone phenomenon that is occasionally observed in lower dilutions of some sera Agglutination titers are ordinarily determined by twofold serum dilutions, and the titer is taken as the highest dilution giving definite agglutination

Wassermann antibody is removed from a small volume of serum by absorption with an aqueous suspension of either VDRL antigen or dehydrated beef-heart. The aqueous suspension of VDRL antigen is prepared by precipitating the standard alcoholic solution with five volumes of 0 85% NaCl solution, and by resuspending the precipitate to its original volume in saline The beef-heart suspension is prepared by washing beef-heart powder once in saline and resuspending to a concentration of 3% For the absorption, equal volumes of the absorbing agent and the serum are mixed, incubated for one hour at 37°C, and overnight at 4°C, and then centrifuged to remove the precipitate Such absorbed sera are considered to be diluted 1.2

VDRL antigen and dehydrated beef-heart remove Wassermann antibody from serum equally well, but the beef-heart suspension is somewhat easier to prepare Moreover, it is more economical than the VDRL antigen, and prepared suspensions of it can be stored under refrigeration for several weeks without deterioration

The agglutination-reaction mixtures are incubated in a water bath at 37°C for 18 hours Merthiolate in the treponeme suspension ordinarily prevents bacterial contamination The presence of agglutination is determined by examination of a wet preparation of each mixture under darkfield microscopy It is essential to place a large drop on a slide and to examine only those organisms which are floating free between the slide and coverglass, because the treponemes that adhere to either glass surface can give a false impression of agglutination The degree of agglutination is read as 0, 1, 2, 3, or 4 plus on the basis of the estimated percentage of treponemes in clumps In a low dilution of high-titer sera the organisms frequently form

of antibody in the source animal, and consequent contamination of the suspension with this cross-reacting antibody.

Despite the progress made in the technical preparation of treponeme suspensions, however, there remains the important and difficult problem of the identification and preservation of the essential antigenic constituents of the treponemes. Dr Hardy and his associates have found that a heat-killed merthiolate-preserved suspension of treponemes contains at least two antigenic components, one of which is reactive with the so-called Wassermann antibody Since it has not been possible to prepare treponeme suspensions that are reactive solely with the " specific " antibody in the treponemal agglutination test, the Wassermann antibody is first absorbed from the reactive mixture. This absorption does not appear to reduce the titer of the " specific " antibody.

Technique of the treponemal agglutination test The technical details of the preparation of treponeme suspensions have been described by Hardy & Nell.[20] Source material is produced in rabbits in a manner similar to that employed for the TPI test (see page 138) The body of each testis is inoculated with approximately 2.5×10^7 treponemes in a volume of 0.5 ml On the 2nd or 3rd day after inoculation daily intramuscular injection of cortisone acetate, 6.0 mg/kg of body-weight, is begun and continued until the animals are sacrificed When a firm orchitis has developed, usually by the 12th day after inoculation, the rabbit is anesthetized with 1-2 ml of 6% sodium pentobarbital, and exsanguinated from the carotid artery The testes are removed and minced with scissors, placed in 50 ml of 0.075 M sodium citrate solution, and shaken continuously for 3 hours at 4°C At the end of that time the fluid, which contains a large number of treponemes, is separated from the testicular tissue; an additional 50 ml of citrate solution is added to the testes, and a second extraction is carried out overnight.

At the end of the extraction each suspension of treponemes is centrifuged at 1000 r.p.m for 5 minutes, in order to remove gross tissue particles The organisms are then sedimented by centrifugation at 35 000 G for 30 minutes at a temperature of 4°C in a Spinco model L centrifuge. The supernate is discarded and the organisms are resuspended in saline to a density of approximately 10^9 treponemes per ml The preparation is then heated (see below), after which merthiolate, to a final concentration of 0.01%, is added as a preservative. Finally, 1/20 by volume of 0.1 M phosphate buffer, pH 7.6, is added.

blood appearing in the extraction medium; the use of isotonic sodium citrate as the extraction medium largely eliminates the formation of the

fibrin clots that otherwise frequently occur and enmesh the treponemes when other extraction media are employed; and immediate heating of the resuspended treponemes appears to prevent deterioration during storage.

Satisfactory treponeme suspensions have been prepared with the Nichols and Chicago strains of *T. pallidum*, the Haiti B strain of *T. pertenue*, the Iraq B strain of bejel treponemes, and the cuniculi A strain of *T. cuniculi*; presumably any strain sufficiently virulent to produce a testicular lesion rich in treponemes within a period of 12 days will yield a satisfactory antigen.

Agglutination tests are carried out in 10 mm×75 mm test tubes in a total test volume of 0 2 ml, comprising 0 1 ml of antigen and 0 1 ml of diluted serum Sera are diluted in saline containing 0 005 M ethylenediamine tetracetate (EDTA), adjusted to pH 7 6

For routine diagnostic purposes a 1 10 dilution of serum is employed to avoid a slight prozone phenomenon that is occasionally observed in lower dilutions of some sera Agglutination titers are ordinarily determined by twofold serum dilutions, and the titer is taken as the highest dilution giving definite agglutination

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large, tightly packed aggregates in which the individual treponeme morphology is completely lost; usually, however, the treponemes agglutinate in loose, lace-like clumps (Fig. 8).

Effect of heat upon treponeme suspensions. Treponeme suspensions prepared in the above manner, without heating or with heating at 56-65°C, are agglutinated only very weakly, and to a very low titer, by immune serum from which Wassermann antibody has been removed by absorption, they are, however, agglutinated strongly, and to a much higher titer, by non-absorbed immune serum or by anti-VDRL serum The agglutinability of the treponeme suspension by specific antibody can be markedly increased by aging, or by heating at 83°C.

Treponeme suspensions that have been stored for months under refrigeration slowly become more agglutinable by specific antibody, reaching their maximal agglutinability after 8-10 months Storage for a further 6 months has not caused any demonstrable change, but after this, deterioration has occurred.

Increased agglutinability of a treponeme suspension can be obtained quickly at any time by heating the treponemes to 83°C The length of time that the organisms must be held at this temperature in order to reach the maximal agglutinability is directly related to the age of the suspension at the time of heating Thus, a suspension which is heated after 1 month or less of storage requires 90-120 minutes at 83°C before it becomes fully agglutinable, while a suspension that has been stored for 6 months reaches maximal agglutinability after only 15-30 minutes at 83°C.

The mechanism involved in the increased agglutinability of treponemes upon heating at a high temperature (or upon aging) is difficult to understand, but recent studies with chemically killed treponemes have suggested a possible explanation. Cardiolipin absorbed immune sera strongly agglutinate these chemically killed treponemes, even without heat treatment. When heated to 65°C they become almost completely inagglutinable, but further heating at 83°C restores the capacity for agglutination. It appears that there may be multiple antigens with different heat stabilities, perhaps in different areas of the organism. The same situation is known with many bacteria Particularly relevant in this connexion may be the studies, undertaken by Italian investigators, on the immunologic properties of soluble extracts of the Reiter organism.[12, 13, 51, 52]

Evaluation of the agglutination test The treponemal agglutination test is much simpler to perform than the TPI test, and given equal degrees of specificity the former test would undoubtedly be far more useful At the

FIG. 8. TREPONEMAL AGGLUTINATION TEST

A. DARKFIELD APPEARANCE WITH NEGATIVE SERUM



B DARKFIELD APPEARANCE AFTER AGGLUTINATION IN PRESENCE OF SPECIFIC ANTIBODY



Photographs taken by Dr Hardy

reserved on its specificity and sensitivity. As will appear later in this monograph, however, the agglutination test has already proved to be a useful investigative tool in the study of experimental treponematosis.

Adhesion phenomena as an immune reaction

As a part of the intensive investigation in this laboratory of antigen-antibody reactions in the treponematoses, attention has been directed to adhesion phenomena The first of these studies, reported by Nelson[43] under the title " The immune-adherence phenomenon ", demonstrated that human erythrocytes in the presence of specific immune serum and complement upon centrifugation remove treponemes and other bacterial species from the suspension.

The studies of Nelson originated in attempts to demonstrate phagocytosis of *T pallidum in vitro* by the technique of Maaløe.[25] During the course of those studies it was postulated that treponemes sensitized with antibody from syphilis serum adhere to the surface of normal human erythrocytes in the presence of complement Furthermore, subsequent experiments suggested that this reaction was an important, even perhaps an essential, precursor to phagocytosis of the treponemes by human leukocytes

The phenomenon was demonstrated by Nelson using the following technique Treponemes were obtained from testicular syphilomas by the same method as used in the TPI test and in the preparation of agglutinating antigens Seven-tenths ml of a suspension containing approximately 10 000 000 treponemes per ml was mixed with 0 1 ml of (*a*) normal rabbit or human serum, and (*b*) varying dilutions of syphilitic rabbit or human serum. After incubation for 30 minutes at 37°C, 0 2 ml of normal human blood was added to each mixture After an additional 30 minutes' incubation the blood cells were removed by centrifugation at low speed (500 r p m for 5 minutes) The number of treponemes in the fluid phase was then counted by darkfield microscopic examination of 0 01 ml of supernatant fluid placed under a 22-mm² coverslip at approximately 675 X magnification.

In this system normal rabbit or human serum was unable to cause disappearance of treponemes from the fluid phase, while syphilis serum was highly active in dilutions up to 1:35. Complement could be supplied through either unheated human serum or unheated guinea-pig serum.

In instances where treponemes disappeared from the fluid phase, they could be recovered from the sediment after freezing and thawing to destroy the red blood cells. Introduction of leukocytes into the immune-serum [illegible] emes recover- [illegible] , reflected the [illegible] tized Essentially this same phenomenon was observed by Nelson with other micro-

organisms, including *Diplococcus pneumoniae*, *Shigella paradysenteriae*, *Salmonella typhi*, *Micrococcus aureus* and *Mycobacterium tuberculosis*.

Attempts have been made to apply the " immune-adherence test " to the assay of specific antibody in treponemal infections and, in turn, to the delineation of diagnostic problems in the same manner as in the TPI test The test has been studied by Daguet & Borel,[11] and Olansky, Harris & Casey,[47] but so far only preliminary data are available concerning its usefulness in these fields.

More recently Lamanna & Hollander [21] have observed that a wide variety of particulate material including a number of bacteria and yeasts, blood platelets, and collodion particles can adhere to *T pallidum* in the presence of immune serum and complement These adhesions, as well as the red-cell " disappearance " noted by Nelson, were considered to be examples of the so-called Rieckenberg reaction

Adhesion was demonstrated with suspensions of the Nichols strain of *T pallidum* obtained from testicular syphilomas by the methods employed by Hardy & Nell [20] in the preparation of treponemal agglutinating antigen The organisms in citrate solution were sedimented by centrifugation and resuspended in fresh citrate solution, recentrifuged, drained carefully to remove the citrate and finally resuspended and stored in sterile 0 85% NaCl solution. All these procedures were carried out at 4°C

One-tenth ml of a suspension of treponemes containing approximately 20 000 000 organisms per ml was then mixed in a small tube with a similar amount of a suspension containing the particulate material suspended in physiological saline, 0 1 ml of fresh guinea-pig serum, 0 1 ml of the serum dilution to be tested, and 0 1 ml saline The mixture was incubated at 34°C for 2 hours As a control, for each serum, the guinea-pig serum was replaced by normal saline.

The mixture was examined under the darkfield microscope The concentration of particulate material (bacteria, etc) was such as to yield about 10-20 clearly distinguishable organisms per oil immersion field, while the concentration of treponemes yielded about 3 per field

Among the particulate material shown to adhere to *T pallidum* in the presence of serum from syphilis patients and complement are the following: *Escherichia coli*, *Alkaligenes faecalis*, *Streptococcus pyogenes*, *Streptococcus lactis*, and *Spirillum rubrum*, erythrocyte ghosts and blood platelets from man, the rabbit and the guinea-pig, and collodion particles Two yeasts, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, showed a low order of adhesion.

Studies on a small group of sera, using streptococci as the particulate material, indicated correlation between this adhesion reaction and serological tests for syphilis In positive reactions more than 10% of the treponemes examined showed 2 or more adhering particles, while in negative reactions less than 10% had adherent particles, usually only 1 or 2.

Apparently Wassermann antibody as well as the more specific types of antibody can give adhesion, since 2 antisera prepared in rabbits against cardiolipin gave positive reactions, but of 7 supposedly BFP reactors, 6 gave negative reactions. Syphilitic sera rendered negative to standard serological tests by absorption with cardiolipin remained adhesion positive.

Lamanna & Hollander [21] noted that the adhesion of blood platelets to pathogenic treponemes in the presence of syphilitic serum was first reported by Krantz [23] in 1930 in a paper which had hitherto gone unnoticed

Antibodies to soluble extracts of pathogenic treponemes

Numerous investigators over the years have attempted to obtain immunologically active fractions from pathogenic treponemes. These attempts have encountered the prime difficulty of separating a relatively small treponemal mass from the much greater mass of animal tissues in which the spirochetes have been propagated [a] Recently, improved methods of extraction and concentration of treponemes have developed to the point where some hope can now be entertained that satisfactory fractionation procedures can be carried out.

In an investigation of this nature, Portnoy & Magnuson [50] have isolated a soluble fraction which seems to be selectively active by complement-fixation techniques.

Treponemes are extracted from syphilomas of rabbits' testes in a manner similar to that described previously, employing sodium citrate solution. The treponemes are concentrated by centrifugation at 20 000 G for one hour, and are then extracted, twice with acetone and twice with ether After the final ether extraction, the sediment is stored *in vacuo* over sulfuric acid in the dark at room temperature For extraction of antigen, 1 ml of desoxycholate solution (0 2% sodium desoxycholate, 0 1 M sodium citrate, and 0 1 M sodium chloride dissolved in distilled water and sterilized by boiling over a low flame for 2 minutes; pH 7 4-7.5) is added for every 5 mg of dried powder, and the suspension is homogenized with syringe and needle After 1 hour's extraction, using a mechanical rotator, the suspension is centrifuged at 800 G for 15 minutes and the supernatant saved This is placed in a cellophane sac and dialysed against M/7 NaCl for 48 hours, with several changes of solution. After dialysis the contents of the sac are centrifuged at 20 000-25 000 G and the supernatant, which contains the active principle, is stored at −20°C until used as antigen in the complement-fixation test

[illegible] methylene blue [illegible] 956) [illegible] anti- [illegible]

The antigen on chemical analysis contained 1.7%-2.0% phosphorus; 7.3% nitrogen; 21-30 mg per cent. total protein. Pentose, glucose and fructose were not detected. The antigen was used in a dilution of 1:5 in the complement-fixation test. Extracts of normal rabbits' testes and extracts of cultures of the Reiter spirochete failed to yield antigens similar to that extracted from syphilitic testes. Absorption of syphilitic sera with VDRL cardiolipin antigen removed antibody that reacted with standard lipoidal antigens, but left undiminished the antibody reacting with the soluble treponemal antigen.

Employing this soluble antigen in the conventional complement-fixation tests, preliminary clinical tests indicate good but not complete agreement with the results of the TPI test, although in early syphilis the "specific" complement-fixation test appeared to be more sensitive than the TPI test, and less sensitive in late and latent syphilis.

Antibodies to soluble extracts of cultivated treponemes

Little effort has been directed toward the extraction of antigens from cultivated treponemes, because long ago they appeared to be immunologically distinct from the pathogenic treponemes. Recent work, particularly by the Italian investigators, however, suggests that the cultivated strains may have antigens in common with the pathogenic strains, and that these antigens may have some diagnostic value.

Gelperin[18, 19] in 1947 and 1948 st[illegible] Reiter treponeme [illegible] digestion were is[illegible] tion by the addition of two volumes of absolute alcohol to the supernatant of high speed centrifugation; and M fraction by the addition of two more volumes of absolute alcohol to the supernatant of the L fraction. Quantitative precipitin analyses were carried out to study their relationships. The results of these analyses are recorded in Table XXXVII. Fractions K and L were serologically active to a slight degree with normal rabbit and human sera, but to a measurably greater extent with sera from rabbits and humans [illegible]

We have no personal experience with the more recent studies carried on in Italy with cultivated strains, and our information is based on the available summaries of this work published in English by D'Alessandro & Dardanoni,[12] D'Alessandro, Oddo & Dardanoni,[13] and by Puccinelli.[51, 52]

These workers have identified three major antigens in the Reiter organism. One is a thermolabile protein, destroyed by heating at 76°-78°C for one hour, or by proteolytic digestion. It is prepared from suspensions of treponemes by freezing and thawing followed by dialysis against distilled

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[illegible] methylene blue [illegible] (1956) [illegible]

Skin reactions with treponemal antigens

Many years ago Neisser (see Meirowsky [39]) investigated skin reactions in normal and syphilitic individuals using syphilitic organ extracts as the antigen. This antigen was obviously crude in the sense that treponemal material comprised only a small fraction of the total antigenically active substances injected. As might have been expected, a disconcertingly high proportion of normal persons showed positive skin reactions, although, at the same time, positive reactions occurred regularly in persons with gummatous types of syphilitic lesions.

Later, Noguchi [44] used an emulsion of cultured spirochetes as a test antigen, again without impressive results from the standpoint of specificity, although this is not surprising in view of the now known lack of antigenic similarity between cultured treponemes and the pathogenic variety.

In recent years a preparation, designated Luotest, has been available commercially. This material is prepared from rabbit syphilomas and contains, in addition to killed *T pallidum*, a large amount of rabbit testicular tissue

With the development in this laboratory of new techniques for the extraction and concentration of pathogenic treponemes it became possible to prepare treponemal antigens of a higher degree of " purity ". Marshak & Rothman,[37] working with such a preparation, in which *T. pallidum* had been killed by 1% formalin, found that 6 normal persons and 6 patients with secondary syphilis gave negative skin tests, while 3 patients with gummas and 8 of 9 patients with pre-natal syphilis gave strong tuberculin-type reactions. The actual skin-test dose contained approximately 20 000 000 treponemes in 0 1 ml.

More recently Csonka,[10] employing a treponemal antigen prepared by Hardy & Nell [20] for use in the treponemal agglutination test, has obtained interesting results in a series of 51 patients with syphilis and 13 normal persons who served as controls. The treponemes in this antigen had been killed by heating to 65°C for 2 hours, with 0.01 % merthiolate added as a preservative. The suspension contained approximately 20 000 000 treponemes in 0 2 ml, the test dose injected. Reactions, which were of the delayed tuberculin-type, were read at 24 and 48 hours and at 7 days when possible. Readings were recorded as negative, ±, +, and ++, a positive reading indicating the presence of an indurated papule at least 5 mm in diameter surrounded by an erythematous halo. Erythema of at least 10 mm in diameter without induration was read as doubtful (±).

[illegible]

25 patients with various forms of late syphilis, 18 were positive and 5 doubtful. Thirteen persons who had no evidence of syphilis gave negative tests.

TABLE XXXVII AMOUNT OF NITROGEN PRECIPITATED FROM 1 ML OF NORMAL AND OF SYPHILITIC HUMAN SERUM AND RABBIT SERUM BY ADDITION OF FRACTIONS OBTAINED FROM TRYPTIC DIGESTION OF REITER TREPONEMES *

Serum source	Amount of nitrogen (N) precipitated according to quantity of fraction added (μg)			
	K 1 5	K 7 4	L 5 6	L 22 5
I Normal rabbit	3 7	9 2	1 3	6 4
II ,,	1 1	5 4	0 8	3 2
920 ,,	0 8			
921 ,,	2 1			
922 ,,	3 8			
923 ,,		6 8		0 0
924 ,,		2 7		0 7
A Normal human	0 0	2 9	0 0	0 0
B ,,	2 8	8 2	1 0	0 0
C ,,	1 7	5 5	0 1	2 5
920 Syphilitic rabbit	3 5			
921 ,,	7 2			
922 ,,	8 1			
923 ,,		9 7		2 9
924 ,,		27 8		14 3
D Syphilitic human	6 9	16 2	5 7	2 3
E ,,	5 5	12 1	3 4	2 6
F ,,	8 0	10 0	0 0	2 2

* Adapted from Gelperin [19]

water. The second is a thermostabile polysaccharide, resisting heating at 100°C for one hour, and digestion by proteolytic enzymes. A third is extracted with ether and is identified with the Wassermann antigen This antigen seems to be absent from some strains of Reiter treponemes Antibodies to both the thermolabile antigen and the thermostabile antigen appear with clinical syphilis infection and both antigens are considered to be specific. The antibody to the thermolabile protein persists for a long time, but the antibody to the thermostabile polysaccharide may be absent in latent syphilis. D'Alessandro & Dardanoni [12] noted that since the protein antigen concerned would have been denatured in the isolation method used by Gelperin [19] the fractions isolated were of a different nature.

agglutinating titer remains essentially unchanged.[6, 20, 45] Because of the technical simplicity of the treponemal agglutination test perhaps the most definitive results have been demonstrated with this technique As many as five absorptions with crude beef-heart antigen have been made by our associates, Dr Paul Hardy and Miss Ellen Nell, without demonstrating a reduction in agglutinating titer, whereas one absorption is usually sufficient to render a serum negative to the VDRL test or to other standard serological tests.

Unfortunately, the difficulty of producing washed and concentrated pathogenic treponemes in quantities sufficient to conduct absorption of the antibodies other than that to cardiolipin is so great that no definitive experiments have been made.

Another approach to this question of the existence of more than one antibody in the serum of treponematosis patients has been through the inoculation of rabbits with aggregates composed of VDRL antigen and human Wassermann antibody Such rabbits, after repeated injections of these antigenic complexes, develop antibodies which are reactive in relatively high titer with lipoidal antigens used in standard serological tests, but do not develop antibodies which react specifically with the treponeme in either the agglutination or the immobilization test [20]

Data relating to the divergence of antibody patterns during the evolution of experimental syphilis will be presented later (see page 158)

The nature of Wassermann antibody. A great mass of clinical and experimental evidence attests to the essential clinical specificity of the standard serological tests which detect Wassermann antibody It is well known that exceptions to this general rule occur, however, so that questions remain concerning the strict biological specificity of these reactions

Among the many theories advanced to explain the occurrence of Wassermann antibody, two have perhaps received the most attention The one, advanced by Eagle [15] and other investigators, holds that Wassermann antibody is a true antibody to a lipoidal component of the treponeme. There is much circumstantial evidence supporting this theory, among which is the remarkable clinical specificity mentioned above. Objections to this theory arise from the fact that virtually any animal or human tissue can serve as a satisfactory source for this antigen, and that quite clearly some persons—the so-called biologic false positive reactors (BFP)—, although free from treponemal infection may nevertheless have Wassermann antibody in their sera

The other theory concerning the biological basis of the standard serological tests, advanced principally by Sachs, Klopstock & Weil,[51] is that treponemal infection causes the breakdown of host tissue and the release of tissue haptens, which are activated by spirochetal protein to form a complete antigen. Supporting this hypothesis is the fact that a number of

Tests made in 5 positive cases with undiluted antigen showed lesser degrees of reaction when the antigen was diluted 1·10 and 1:100. Five patients who were positive to the treponemal antigen were simultaneously tested with three unrelated materials—Frei antigen, Dmelcos vaccine, and sterile pus—with negative results in each instance.

These rather preliminary results with relatively highly concentrated treponemal antigens suggest that on the whole the reactions obtained were more clear-cut, and perhaps more specific, than those to be expected with the older antigens. The results likewise offer some support to the long-held opinion that in the late stages of syphilis many patients acquire some degree of hypersensitivity to the treponeme, and that this phenomenon provides the basis for the clinical and histopathological characteristics of the gumma and the late manifestations of syphilis.

Significance of the Newer Serological Tests Employing Treponemes as Antigens

It will not be our primary purpose here to attempt a clinical evaluation of the serological tests in which treponemes or fractions of treponemes are employed as antigens. We shall, however, endeavor to examine certain fundamental questions raised by the development of these tests, in an attempt to appraise their biological and perhaps their clinical significance.

One antibody or more? An important basic question is whether treponemal antigens are capable of detecting and measuring a different antibody from that commonly measured in the standard serological tests employing lipoidal antigens. The weight of evidence strongly indicates that at least two distinct kinds of antibody develop in response to treponemal infection in either man or animals, one reacting primarily with tissue extracts of a lipoidal nature, the other reacting with another, as yet unidentified, component of the treponeme This conclusion is arrived at on the basis of (*a*) absorption experiments: (*b*) the absence of " treponemal antibodies " in immune serum produced by the inoculation of animals with lipoidal flocculates, and (*c*) the divergence of the patterns of the two antibodies during the evolution of experimental syphilis.

Most serum specimens from human beings or animals infected with one of the pathogenic treponemes give positive reactions to all the standard serological tests, and to all tests in which pathogenic treponemes constitute the antigen, whether the test is based on immobilization, agglutination, or adhesion of particulate material. It has repeatedly been shown, however, that absorption of these sera with lipoidal antigens, such as the VDRL cardiolipin antigen, or with a crude powder of beef-heart, will render the sera negative to standard serological tests, while the immobilizing and

stimulate the production of trace amounts of Wassermann antibody, thus giving rise to tests which are considered to be falsely positive, although in an immunological sense the positive test may be entirely valid

The nature of immobilizing and other treponemal antibodies. Both laboratory and clinical evidence lead to the conclusion that immobilizing antibody may be regarded, in the strict immunological sense, as an antibody to pathogenic *Treponema* This antibody is induced in both man and laboratory animals by all of the pathogenic treponemes studied thus far, and in that sense may be regarded as a response to a group specific antigen. Immobilizing antibody appears to have a high level of specificity, as determined by laboratory observations and exhaustive clinical study It would perhaps be going too far to conclude that the presence of immobilizing antibody always and without exception reflects prior contact with one or another variety of pathogenic *Treponema,* but the evidence indicates that this is almost always the case

It is not known whether the entire treponeme or a particular component of the treponeme is responsible for the production of immobilizing antibody, for no one has as yet induced this antibody with a fraction of treponemal organisms

The relationship of immobilizing to agglutinating and other "specific" antibodies As outlined above, infection with one of the treponemal organisms induces the production by the host of antibodies which can react directly with the treponeme or with one of its chemical components *in vitro* Thus far antibodies have been found which, under the appropriate technical conditions, immobilize treponemes,[45] induce agglutination of treponemes,[20] cause the adhesion to treponemes of certain particulate materials in the mixture,[21, 43] and bring about the fixation of complement in the presence of a chemical fraction of *T pallidum* [50] It is not known with certainty whether these phenomena are all produced by the same antibody or whether more than one antibody is involved.

When a large series of sera are compared according to the results of the immobilization and the agglutination test, as has been done in our own laboratory by Dr Hardy and Miss Nell (Table XXXVIII), the results of the two tests agree in the vast majority of instances There are disagreements, however, and these are difficult to explain It is our impression that the agglutination test as performed in this laboratory is somewhat more sensitive than the TPI test from a purely quantitative standpoint, and some of the differences noted may be explained on this basis.

It may be, however, that distinct antibodies are responsible for immobilization on the one hand and agglutination on the other. Since both antibodies are clearly induced by some component of the treponeme it is to be expected that the two antibodies will occur together in most sera, and such

acute and chronic infections, other than those induced by treponemes, frequently invoke the production of Wassermann antibody, thus giving rise to biologic false positive tests Moreover, as mentioned above, Wassermann antibody can be artificially invoked in animals by injection of tissue lipoidal aggregates

In recent experiments in this laboratory, Osler & Knipp (unpublished observations, 1955), using precise quantitative precipitin analyses, found that human Wassermann antibody reacts equally well with human or bovine cardiolipin and almost as well with sitolipin from wheat germ. These data, as well as the heat lability of the Wassermann antibody as compared with that of immobilizing and agglutinating antibody, lead Osler to favor the auto-antibody hypothesis as the mechanism for the formation of Wassermann antibody. The data *per se* do not exclude the possibility that cardiolipin is an antigenic component of the treponeme, and indeed the authors of this monograph incline toward the view that Wassermann antibody in treponemal disease is formed in response to direct stimulation by a component of the treponeme itself Many investigators have observed that the titer of Wassermann antibody can be positively correlated with the number and extent of syphilitic lesions More recently, this observation has been quantitatively documented by Schipper & Chesney [56]

The clinical significance of biologic false positive serological tests While tests for Wassermann antibody have been, and still are, enormously useful, their role must be reassessed in relation to tests for other types of antibody

It is well known that many acute infections, such as pneumonia, influenza, vaccinia, malaria, and a host of others, may give rise to a substantial increase in Wassermann antibody in the serum of the affected patient.[40] The titer of the antibody under these circumstances commonly reaches its peak within 3 weeks of the onset of the illness and then gradually declines over a period of 1-2 months

More obscure from both a clinical and a biological standpoint are patients who apparently maintain a substantial titer of Wassermann antibody over a period of months or years in the almost certain absence of treponemal infection. Some of these patients have been discovered to have one of the so-called collagen diseases, such as lupus erythematosis, or rheumatoid arthritis, and it has been recognized [41] that other such patients may be in an incipient stage of one of these diseases However, in a considerable proportion of so-called BFP reactors no underlying disease processes can be identified. When a serum contains as little as 0 3 μ of Wassermann antibody N per ml, a positive STS may be obtained.[48] Since there are undoubtedly many phospholipids whose chemical structure resembles that of cardiolipin, it is not surprising that a variety of disease incitants may

bial antigen-antibody systems, it would seem likely that a mosaic of antigens is present in the treponeme and that this invokes a corresponding mosaic of antibodies in the host. Nevertheless, even assuming that distinct antibodies exist, it is to be expected that, in general, they will tend to occur together when the whole living treponeme provides the antigenic stimulus.

The evidence thus far indicates that the antibody detected by treponemal antigens not only is distinct from Wassermann antibody, but also rarely if ever occurs in the absence of specific antigenic stimulus by pathogenic treponemes Judgement must be reserved on whether it *never* occurs except under those circumstances. From a strictly immunochemical point of view, and given the antigenic complexity of living matter, the chances seem good that non-treponemal antigenic material may at times (if only extremely rarely) duplicate a portion of the antigenic mosaic of the treponeme and thus invoke false positive tests

We come, therefore, to a situation governed not by absolutes, but by the laws of probability. How then may these phenomena be utilized to illuminate the practical problems relative to the treponematoses in man?

Criteria for the evaluation of serological tests From the foregoing discussion it is evident that serological tests in the treponematoses may be evaluated from the standpoint of their biological specificity, or from the point of view of their clinical specificity. With respect to the former question, the problem resolves itself essentially into a study of the antigenic components of the treponemes in relation to the occurrence of specific antibodies to those components

At the clinical level, however, the whole treponeme provides the antigenic stimulus, with perhaps multiple antibody responses corresponding to the complexity of the antigenic stimulus The practical question, therefore, is. Which of these antibodies provides the most definitive index of treponemal infection? Is there any one antibody or any one test that clearly enjoys sufficient biological and clinical certitude to justify its acceptance as the standard by which all other tests are judged?

As biologists, we must answer this question in the negative As clinicians, faced with the practical necessity of making day-to-day decisions, we may advance a tentative and not too satisfactory answer It is evident that in the vast majority of individuals with treponemal infections all the tests referred to in this section are positive On the other hand, some individuals (and experimental animals, too) known to be infected with one or another of the treponemal organisms exhibit negative standard serological tests and positive tests with treponemal antigens Moreover, a small proportion of all persons observed in the usual clinic show positive standard serological tests in face of overwhelming clinical and epidemiological evidence that they have never been infected with one of the treponemal group of organisms, the TPI test and the TPA test are usually negative in such individuals.

TABLE XXXVIII CORRELATION OF RESULTS OF IMMOBILIZATION, AGGLUTINATION AND STANDARD SEROLOGICAL TESTS ON PATIENTS WITH SYPHILITIC AND NON-SYPHILITIC CONDITIONS *

Serological tests			Clinical categories of patients					
Agglutination	TPI	STS [a]	Normal or non-syphilitic disease	Presumably BFP [b]	Secondary syphilis	Latent syphilis	Treated late syphilis	
							CNS	Other
Positive	Positive	Positive			13	78	73	29
"	"	Negative				11	17	2
"	Negative	Positive		4			3	
Negative	Positive	.				6	2	1
Positive	Negative	Negative					5	
Negative	Positive	,					1	
"	Negative	Positive		99			1	
"	"	Negative	76				1	
Total			76	103	13	95	103	32

* From data supplied by Hardy and Nell
[a] Flocculation test with VDRL antigen
[b] BFP = Biologic false positive standard serological test

seems to be the case; but, given that they are distinct antibodies, it would not be unreasonable to expect that substantial differences between their respective proportions in the sera might occur in occasional specimens The differences detected by Hardy and Nell in the response of some sera to heated and unheated treponemal suspensions, as mentioned above, suggests that antibodies to different components of the treponeme can be detected with techniques of this character. The experiments of Portnoy & Magnuson [50] likewise show that antibodies to a particular chemical component of the treponeme do arise during the course of syphilitic infection, and there are indications that these antibodies are not necessarily identical with those active in the TPI test

Some of the comparative studies using two or more of these tests on the same serum specimens performed both in our laboratory and by Chacko [8] are shown in Tables XXXVIII-XLII. It is evident that during the course of treponemal infection, antibodies to one or more antigenic components of the treponeme develop, and may be detected by *in vitro* tests with the treponeme itself, or with one of its chemical components, as the reacting antigen. It is not yet clear whether or not the various techniques for demonstrating *in vitro* interaction with the treponeme, or with its components, detect the same antibody. By analogy with what is known of other micro-

While there is some variation from rabbit to rabbit, in general the titer of immobilizing, agglutinating and Wassermann antibodies rises during the early days of active lesions; immobilizing and agglutinating antibodies then continue at a high level for many months, even after the syphilitic lesions

TABLE XXXIX COMPARISON OF TITERS OF IMMOBILIZING ANTIBODY AND WASSERMANN ANTIBODY (VDRL TEST) IN RABBITS WITH EXPERIMENTAL SYPHILIS

Months after infection	Rabbits inoculated intratesticularly[a]										Rabbits inoculated intracutaneously[b]					
	JH1		JH5		JH7		JH10		JH20		16 17		15-73[c]		16 75	
	TPI	VDRL	TPI	VDRL	TPI	VDRL	TPI	VDRL	TPI	VDRL	TPI	VDRL	TPI	VDRL	TPI	VDRL
1	40[d]	4	25	32	±	32	75	64	0	0	±	±	0	0	±	0
2	100	32	60	4	160	4	190	16	—	—	40	8	30	8	100	4
3	80	8	60	+	170	+	—	—	20	32	180	16	140	8	220	16
4	85	4	125	±	190	±	—	—	80	16	—	—	—	—	—	—
5	160	4	180	±	160	±	350	8	160	4	—	—	—	—	—	—
6	190	2	180	±	270	±	300	±	110	2	900	8	460	+	150	0
7	—	—	200	+	320	0	520	±	40	±	—	—	—	—	—	—
8	300	2	—	—	—	0	460	±	110	±	—	—	—	—	—	—
9	320	+	320	±	—	—	—	0	—	—	920	4	740	1	640	1
10	300	+	180	±	—	—	—	0	—	—	—	—	—	—	—	—
11	640	+	320	±	—	—	—	—	—	—	—	—	—	—	—	—
12	360	±	—	—	—	—	—	—	—	—	640	2	820	0	740	0
Lesions developed	—		—		—		—		—		20 days		22 days		20 days	
Lesions healed	—		—		—		—		—		120 days		100 days		100 days	

[a] From Chacko[4]
[b] TPI tests by Nelson in our laboratory
[c] Data for this animal also shown in Fig 9
[d] Numbers denote reciprocal of dilution, + = positive, ± = weak positive — = no test

have regressed and healed The titer of Wassermann antibody on the contrary tends to parallel the activity of the lesions, and to decline as the lesions subside. (See also Table XLI)

If curative treatment is given relatively early during the course of the infection, e g at 2 months, the titer of both immobilizing and agglutinating antibody declines, although the latter rarely reaches zero The titer of

It appears, therefore, that tests employing treponemal antigens have a higher degree of specificity than those in which tissue lipoidal extracts are used as antigens Of the former tests, the TPI has been more thoroughly studied in relation to good clinical and epidemiological data than the other tests in which treponemes or fractions of the organism are used as antigens However, the data indicate that there is good agreement between the agglutination and the immobilization tests. In cases where the two tests are in conflict it is too early to say whether one is necessarily more accurate than the other.

A conclusion to be drawn from this discussion is that the evaluation of a given serological test must, in the final analysis, be based squarely on the diagnoses of individual patients, as determined by exhaustive clinical and epidemiological investigations. To evaluate a new serological test in this manner is obviously a major undertaking, and can be accomplished only in well-organized and competently staffed clinics. For the initial evaluation of a particular test it is essential to have sera available from one series of patients in which it is reasonably certain that treponemal infection has taken place, and from another series of patients in which it is equally certain that treponemal infection has not taken place With these as starting points some judgement can be made concerning the uses and limitations of the tests under assay

Antibody Patterns During the Course of Experimental Syphilis

We have many data indicating the general pattern of antibody responses in syphilis as determined by the TPI test, the agglutination test, and standard serological tests, particularly the VDRL flocculation test The most definitive observations have been made on experimentally infected rabbits, but the data on syphilitic patients suggest that the pattern is similar in its essential features to that in the experimental disease

The observations that will be cited here were made principally on rabbits, inoculated intratesticularly at the same time with aliquots of the same emulsion of Nichols strain treponemes Groups of these animals were then given curative penicillin treatment and were later subjected at varying intervals to challenge inoculation by the intracutaneous route with the homologous strain of treponeme. The results of challenge inoculation of these groups have previously been given in Table XXXIII, page 127.

The pattern of antibody titers in animals observed for a year or more without treatment are shown in Tables XXXIX and XL, and the titers for one animal are shown graphically in Fig. 9 Observations made by Chacko[6] in London are also included in Table XXXIX. Titers are computed on the basis of the final concentration of the serum in the test mixture, except in the case of the VDRL titers, which are expressed in terms of the dilution of serum placed in the test mixture.

TABLE XLI. PATTERN OF ANTIBODY TITERS FOLLOWING CURATIVE TREATMENT AND CHALLENGE INOCULATION FIRST INOCULATION, TESTICULAR, CHALLENGE INOCULATION, INTRACUTANEOUS (BOTH NICHOLS STRAIN) *

Time	Rabbit 18 14			Rabbit 18-16			Rabbit 18 18			Rabbit 19 21		
	TPI	Agglutination	VDRL	TPI	Agglutination	VDRL	TPI	Agglutination	VDRL	TPI	Agglutination	VDRL
2 months post inoculation	1 280	2560	32	420	640	32	440	640	64	230	640	8
2 months post treatment	—	160		—	160		—	160		—	160	
4 " "	—	320		—	160		—	160		—	160	
6 " " "	290	—	0	32	80	1	40	160	0	14	160	0
9 " " "	—	—	0	—	320	0	—	160	0		80	0
12 " " "	46	160	0	±	—	0	0	—	0	0	160	0
2 weeks post challenge	26	40	0	±	—	1	0	—	1	14	160	0
4 weeks post challenge	1 900	320	—	90	160		140	320	—	220	80	—
Lesions developed	10 days			14 days			10 days			10 days		
Lesions healed	70 days			72 days			80 days			80 days		
Lesions upon challenge	+++			+++			++			++		

* Animals belong to Group B, (Table XXXIII), treatment 2 months post inoculation, challenge 12 months post treatment.

Wassermann antibody declines rapidly after treatment (Table XLI) We have only limited observations on animals treated late in the course of the infection, but what data there are indicate that the titer of immobilizing and agglutinating antibody declines much more slowly if at all under those circumstances. On the whole, the evidence indicates that, as performed in this laboratory, the agglutination test is more sensitive in detecting small amounts of antibody than the TPI test.

Data on antibody patterns during the course of other experimental treponemal infections indicate that the essential features are similar to those observed in experimental syphilis.

Relationship of Treponemal Antibody to Resistance

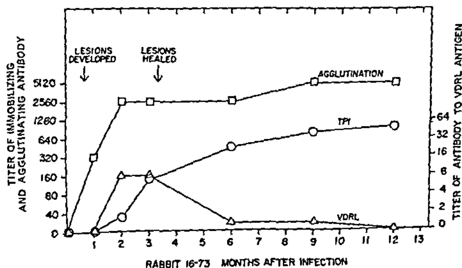
Antibodies to various components of a bacterial cell may or may not play a conspicuous role in the resistance to that particular disease. However, even in the case of antibodies clearly associated with resistance, this

TABLE XL. COMPARISON OF AGGLUTINATION AND VDRL TITERS FOLLOWING TESTICULAR INOCULATION WITH NICHOLS STRAIN*

Time after infection	Rabbit 18-46		Rabbit 18 52		Rabbit 18 53	
	Agglutination	VDRL	Agglutination	VDRL	Agglutination	VDRL
Pre-inoculation	0	0	0	0	0	0
3 weeks	640	16	640	256	320	256
1 month	5 120	16	640	256	1 280	128
2 months	2 560	16	2 560	256	5 120	32
3 months	2 560	2	2 560	16	1 280	8
5 months	—	—	5 120	8	640	8
6 months	2 560	1	1 280	4	1 280	1
14 months	5 120	1	2 560	—	640	1
Lesions developed	10 days		7 days		10 days	
Lesions healed	90 days		150 days		150 days	

* No treatment was given Rabbits were unchallenged controls of experiment shown in Table XXXIII

FIG. 9 ANTIBODY PATTERNS FOLLOWING INTRACUTANEOUS INOCULATION OF RABBITS



TPI, TPA and STS titers on a single rabbit (No. 16-73) after the intracutaneous inoculation of Nichols strain treponemes

TABLE XLI PATTERN OF ANTIBODY TITERS FOLLOWING CURATIVE TREATMENT AND CHALLENGE INOCULATION FIRST INOCULATION, TESTICULAR; CHALLENGE INOCULATION, INTRACUTANEOUS (BOTH NICHOLS STRAIN) *

Time	Rabbit 18 14			Rabbit 18 16			Rabbit 18 18			Rabbit 18 21		
	TPI	Agglutination	VDRL	TPI	Agglutination	VDRL	TPI	Agglutination	VDRL	TPI	Agglutination	VDRL
2 months post inoculation	1 280	2560	32	420	640	32	440	640	64	230	640	8
2 months post treatment	—	160		—	160		—	160		—	160	
4 " "	—	320		—	160		—	160		—	160	
6 " " "	230	—	0	32	80	1	40	160	0	14	160	0
9 " " "	—	—	0	—	320	0	—	160	0		80	0
12 " " "	46	160	0	±	—	0	0	—	0	0	160	0
2 weeks post challenge	26	40	0	±	—	1	0	—	1	14	160	0
4 weeks post challenge	1 900	320	—	90	160		140	320	—	220	80	—
Lesions developed	10 days			14 days			10 days			10 days		
Lesions healed	70 days			72 days			80 days			80 days		
Lesions upon challenge	+++			+++			++			++		

* Animals belong to Group B, (Table XXXIII), treatment 2 months post inoculation, challenge 12 months post treatment.

Wassermann antibody declines rapidly after treatment (Table XLI) We have only limited observations on animals treated late in the course of the infection, but what data there are indicate that the titer of immobilizing and agglutinating antibody declines much more slowly if at all under those circumstances. On the whole, the evidence indicates that, as performed in this laboratory, the agglutination test is more sensitive in detecting small amounts of antibody than the TPI test

Data on antibody patterns during the course of other experimental treponemal infections indicate that the essential features are similar to those observed in experimental syphilis.

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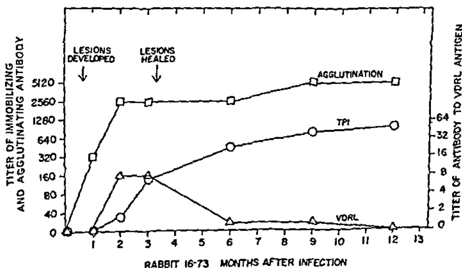
Antibodies to various components of a bacterial cell may or may not play a conspicuous role in the resistance to that particular disease. However, even in the case of antibodies clearly associated with resistance, this

TABLE XL. COMPARISON OF AGGLUTINATION AND VDRL TITERS FOLLOWING *TESTICULAR INOCULATION WITH NICHOLS STRAIN* *

Time after infection	Rabbit 18 46		Rabbit 18-52		Rabbit 18-53	
	Agglutination	VDRL	Agglutination	VDRL	Agglutination	VDRL
Pre inoculation	0	0	0	0	0	0
3 weeks	640	16	640	256	320	256
1 month	5 120	16	640	256	1 280	128
2 months	2 560	16	2 560	256	5 120	32
3 months	2 560	2	2 560	16	1 280	8
5 months	—	—	5 120	8	640	8
6 months	2 560	1	1 280	4	1 280	1
14 months	5 120	1	2 560	—	640	1
Lesions developed	10 days		7 days		10 days	
Lesions healed	90 days		150 days		150 days	

* No treatment was given Rabbits were unchallenged controls of experiment shown in Table XXXIII.

FIG. 9 ANTIBODY PATTERNS FOLLOWING INTRACUTANEOUS INOCULATION OF RABBITS



TPI, TPA and STS titers on a single rabbit (No. 16-73) after the intracutaneous inoculation of Nichols strain treponemes

the infection exhibited rather low titers of both immobilizing and agglutinating antibody, and at the same time showed some but not a high degree of immunity on challenge inoculation

Group C animals on the other hand, which were treated and challenged 6 months after the initial infection, all had high levels of both immobilizing and agglutinating antibody, and at the same time exhibited a high level of immunity upon challenge inoculation In none of these groups, however, was there a good correlation between the degree of immunity and the level of Wassermann antibody. The quantitative TPI tests in these experiments were all conducted by our former associate, Dr Robert A. Nelson, jr., and the quantitative agglutination tests by our associates, Dr Paul H Hardy, jr, and Miss E E Nell The fact that the two tests were run at different times accounts for certain unfortunate gaps in these data

Additional data on the problem of the relationship of immobilizing antibody to immunity were derived from an experiment carried out by Turner & Nelson,[62] and referred to previously in Table XXXIV, page 129. Rabbits were inoculated intrastesticularly with graded doses of treponemes, thus assuring differences in the incubation period of lesions Curative treatment was given simultaneously to all the animals 28 days after the initial inoculation, and all the animals were challenged by intracutaneous inoculation 2 weeks later

The median titer of immobilizing antibody together with the results of challenge inoculation are shown in Table XXXIV Again it will be noted that there appears, in general, to be a direct relationship between the degree of resistance and the titer of immobilizing antibody

Inspection of Table XLI will also reveal what appear to be instances of a booster-type rise in immobilizing antibody following challenge inoculation, there is a suggestion that a rise may also have occurred in agglutinating antibody, but here the data are very limited. This, of course, is a well-known phenomenon in immunology and its observation under these circumstances might not be unanticipated A booster-type rise in immobilizing antibody has likewise been observed by McLeod & Magnuson [29] following the injection of killed treponemes into animals with treated latent syphilis

Magnuson, Thompson & McLeod [35] have studied the question of the relationship of immobilizing antibody to resistance with results which apparently diverge from those obtained in our own laboratory, since they were unable to demonstrate a close correlation of resistance and immobilizing antibody titer. Close inspection of their protocols, however, shows that the majority of animals challenged 12 weeks after treatment had immobilizing antibody at the time of challenge or else developed it within 2 weeks following challenge; none of the animals developed symptomatic infection following challenge It should be noted that challenge inoculation was made intratesticularly and that as mentioned previously

can be established only by the study of groups of human beings or animals, since in an individual animal there may not be a close correlation between antibody titer and the degree of resistance Animals with high titers, however, as a group, usually show higher degrees of resistance than groups with low titers.

Some data on the relation of immobilizing, agglutinating, and Wassermann antibody titers to immunity in syphilis are shown in Table XLII. Rabbits belonging to Groups A and B treated early during the course of

TABLE XLII RELATIONSHIP OF TPI, AGGLUTINATION AND VDRL TITERS TO IMMUNITY FIRST INOCULATION, TESTICULAR, CHALLENGE INOCULATION, INTRACUTANEOUS (BOTH NICHOLS STRAIN) *

Group	Rabbit Number	Antibody titer in various tests at time of challenge inoculation			Results of challenge controls all ++++
		TPI	Agglutination	VDRL	
A	17 78	42	80	8	0
Treatment 3 weeks	17 79	—	40	4	+++
	17-80	250	—	8	++
Challenge 2 weeks later	17 81	60	80	32	++
	17 82	< 20	160	8	0
	17-83	< 20	80	16	+
B_1	17 84	360	10 240	32	0
Treatment 2 months	17-85	56	5 120	16	±
Challenge 2 weeks later	17-86	180	10 240	32	0
	17-88	240	10 240	64	+
B_2	18-04	18	—	0	+
Treatment 2 months	18-06	32	—	1	++
Challenge 12 months later	18 13	46	640	0	+
	18-14	46	160	0	++
	18-16	±	320	1	+++
	18 18	0	160	0	++
	18 21	0	160	0	++
C	18 25	860	2 560	8	0
Treatment 6 months	18 26	860	2 560	1	0
Challenge 2 weeks later	18 28	740	2 560	1	0
	18 29	660	7 280	16	0
	18-30	620	2 560	4	0
	18 31	440	2 560	16	0
	18 33	1 180	5 120	2	0
	18 35	800	5 120	2	0
	18 36	520	5 120	2	0
	18 38	1 300	5 120	2	0
	18-41	1 000	10 240	1	0

* Groups are the same as those shown in Table XXXIII

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development of asymptomatic infection was largely independent of the size of the challenging inoculum.

In further experiments, McLeod & Magnuson [26] injected a suspension of killed *T. pallidum* into rabbits with treated latent syphilis, and noted a rise in the proportion of animals developing a positive TPI test, but no quantitative estimations were made, all the animals developed Wassermann antibody. Upon challenge inoculation 10 of 11 animals developed a lesion as the result of a single intradermal injection of approximately 200 *T pallidum*, and the 11th animal developed an asymptomatic infection. "Immunizing" injections of killed spirochetes were made over a period of 2-7 months, and challenge inoculation was carried out from 1 to 5½ months after the last injection. TPI titers ranged from 1 : 20 to 1 : 200 at the time of challenge; 2 animals had negative TPI tests

McLeod & Magnuson [26] likewise carried out interesting experiments on the induction of positive TPI tests in mice When inoculated with a single intraperitoneal injection of 8 000 000 living *T. pallidum* of the Nichols strain, all of 14 mice became infected, but only 2 developed a weakly positive TPI test When given repeated intraperitoneal inoculations, (30 000 000 treponemes in 7 doses), however, all the mice developed immobilizing antibodies in titers varying from 1 . 80 to 1 . 470. All the mice remained asymptomatic throughout

Both groups of mice were treated with curative doses of penicillin 14 weeks after the initial inoculation, and were then challenged intraperitoneally with 1 000 000 living *T. pallidum* Approximately half the animals in each group, including the controls, became infected, as shown by tissue transfers to rabbits

High-titer immobilizing antibody was likewise induced in mice by intraperitoneal injection of 30 000 000 heat-killed *T pallidum* over a period of 10 days. Titers varied from 1 · 60 to 1 : 270 Mice do not develop demonstrable active immunity as a result of syphilitic infection, while TPI antibody does result from such infection

McLeod & Magnuson [26] interpret the foregoing experiments as further evidence that immobilizing antibody does not play an important role in immunity in syphilis While caution must be exercised in directly comparing TPI titers obtained in one laboratory with those obtained in another, it should be noted that all the titers in the foregoing experiments of McLeod & Magnuson were substantially lower than those observed in our animals exhibiting a high degree of immunity.

It is our opinion that, while there is obviously no close correlation between Wassermann antibody and acquired resistance in experimental syphilis, the limited data with respect to immobilizing antibody indicate that there is a positive correlation between the amount of this antibody and immunity. The same statement can be made for the relation between *agglutinating* antibody and immunity.

41 Moore, J. E. & Mohr, C. F (1952) The incidence and etiologic background of chronic biologic false-positive reactions in serological tests for syphilis Preliminary report, *Ann intern Med*, 37, 1156

42 Nelson, R A, jr (1948) Factors affecting the survival of *Treponema pallidum* in vitro, *Amer J Hyg*, 48, 120

43 Nelson, R A, jr (1953) The immune-adherence phenomenon, *Science*, 118, 733

44 Nelson, R A, jr. & Diesendruck, J A (1951) Studies on immobilizing antibodies in syphilis I Technique of measurement and factors influencing immobilization, *J Immunol*, 66, 667

45 Nelson, R A, jr & Mayer, M M (1949) Immobilization of *Treponema pallidum* in vitro by antibody produced in syphilitic infection, *J exp Med*, 89, 369

46 Noguchi, H (1911) A cutaneous reaction in syphilis, *J exp Med*, 14, 557

47 Olansky, S, Harris, A & Casey, H (1954) Immune adherence test for syphilis, *Publ Hlth Rep (Wash)*, 69, 521

48 Osler, O, Hardy, P H, jr & Sharp, J T (1954) The fixation of complement by human sera and alcoholic extracts of human cardiac tissue, *Amer J Syph*, 38, 554

49 Portnoy, J, Harris, A & Olansky, S (1953) Studies on the *Treponema pallidum* immobilization (TPI) test I The effect of increased sodium thioglycollate and complement, *Amer J Syph*, 37, 101

50 Portnoy, J & Magnuson, H J (1955) Immunologic studies with fractions of virulent *Treponema pallidum* I Preparation of an antigen by desoxycholate extraction and its use in complement fixation, *J Immunol*, 75, 348

51 Puccinelli, V A (1951) Recent advances in the serodiagnosis of syphilis, *Amer J Syph*, 35, 340

52 Puccinelli, V A (1952) Plurality of antibodies in syphilitic serum and clinical practice, *Brit J vener Dis*, 28, 184

53 Reynolds, F W (1941) The fate of *Treponema pallidum* inoculated subcutaneously into immune rabbits, *Bull Johns Hopk Hosp*, 69, 53

54 Sachs, H, Klopstock, A & Weil, A J (1925) Die Entstehung der syphilitischer Blutveränderung, *Dtsch med Wschr*, 51, 589

55 Saurino, V R (1953) The modification of the Nelson treponemal sustaining medium for use in the *Treponema pallidum* immobilization test, *Amer J Syph*, 37, 112

56 Schipper, G J & Chesney, A M (1950) The effect of the method of inoculation on the behavior of the serologic test for syphilis in experimental syphilis of the rabbit, *Amer J Syph*, 34, 25

57 Schobl, O (1930) The duration of anti-treponematous immunity in Philippine monkeys originally conveyed by immunization with killed yaws vaccine, *Philipp J Sci*, 43, 599

58 Schobl, O, Tanabe, B & Miyao, I (1930) Preventive immunization against treponematous infections and experiments which indicate the possibility of anti-treponematous immunization, *Philipp J Sci*, 42, 219

59 Tani, T, Inoue, R & Asano, O (1951) Studies on the preventive inoculation against syphilis, *Jap med J*, 4, 71

60 Turner, T B (1939) Protective antibodies in the serum of syphilitic rabbits, *J exp Med*, 69, 867

61 Turner, T B & Hollander, D H (1952) *Studies on the action of cortisone in experimental syphilis*, New York (New York Academy of Medicine, *Symposia of the Section of Microbiology*, No 6) (Reprinted in *Amer J Syph*, 1954, 38, 371)

21. Harris, A et al (1935) Studies on the *Treponema pallidum* (TPI) test II. Evaluation of quantitative control serums, *Amer J Syph.*, 37, 106

22 Hollander, D H., Turner, T B & Nell, E E (1952) The effect of long continued subcurative doseses of penicillin during the incubation period of experimental syphilis, *Bull Johns Hopk Hosp*, 90, 105 (Reprinted in *Arch med Cuba*, 1953, 4, 26)

23. Krantz, W (1930) Grundsätzliches zur experimentellen Syphilis, *Münch med Wschr*, 77, 1184

24 Lamanna, C & Hollander, D H (1956) Demonstration of particulate adhesion of the Rieckenberg type with the spirochete of syphilis, *Science*, 123, 989

25 Maaløe, O (1946) *On the relationship between alexin and complement*, Copenhagen (Thesis)

26 McLeod, C P & Magnuson, H J (1951) Development of treponemal immobilizing antibodies in mice following injection of killed *Treponema pallidum*, *J vener. Dis Inform*, 32, 274

27 McLeod, C. P & Magnuson, H J. (1952) Penicillin treatment of experimental yaws in rabbits with special reference to criteria of infection and cure, *Amer J Syph*, 36, 545

28. McLeod, C P & Magnuson, H J. (1953) Agglutination of *Treponema pallidum* in syphilitic serums, *Publ. Hlth Rep (Wash)*, 68, 747

29 McLeod, C P & Magnuson, H J (1953) Production of immobilizing antibodies unaccompanied by active immunity to *Treponema pallidum* as shown by injecting rabbits and mice with killed organisms, *Amer J Syph*, 37, 9

30 McLeod, C P & Magnuson, H J (1955) *A study of cross immunity between syphilis and yaws in penicillin-treated rabbits Part II Development of asymptomatic reinfection* (Unpublished working document WHO/VDT/140 presented to the International Conference on Yaws Control, Nigeria, 1955)

31 McLeod, C P. & Stokes, P S. (1955) Agglutination of *Treponema pallidum* by reagin antibody, *Publ Hlth Rep (Wash)*, 70, 379

32 Magnuson, H J., Halbert, S & Rosenau, B (1947) Attempted immunization of rabbits against syphilis with killed *Treponema pallidum* and adjuvants, *J. vener Dis Inform*, 28, 267

33 Magnuson, H J & Rosenau, B J. (1948) The rate of development and degree of acquired immunity in experimental syphilis, *Amer. J Syph*, 32, 418

34 Magnuson, H. J., Rosenau, B J & Clark, J W (1949) The duration of acquired immunity in experimental syphilis, *Amer J. Syph*, 33, 297

35 Magnuson, H J, Thompson, F A & McLeod, C P. (1951) Relationship between treponemal immobilizing antibodies and acquired immunity in experimental syphilis, *J. Immunol.*, 67, 41

36. Magnuson, H J., Thompson, F A & Rosenau, B J (1950) The effect of subcurative penicillin therapy upon the rate of development of acquired immunity in experimental syphilis, *Amer J Syph*, 34, 219

37. Marshak, L C & Rothman, S (1951) Skin testing with a purified suspension of *Treponema pallidum*, *Amer J Syph*, 35, 35

38 Matsumoto, S (1930) *Experimental syphilis and framboesia*, Kioto (*Monographiae Actorum Dermatologicorum*, Series B, *Syphilidologica*, No. 3)

39. Mc [illegible]

40 Mc [illegible]

41. Moore, J E & Mohr, C F (1952) The incidence and etiologic background of chronic biologic false-positive reactions in serological tests for syphilis Preliminary report, *Ann. intern Med*, 37, 1156

42 Nelson, R. A, jr (1948) Factors affecting the survival of *Treponema pallidum* in vitro, *Amer J Hyg*, 48, 120

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32 Magnuson, H J, Halbert, S & Rosenau, B (1947) Attempted immunization of rabbits against syphilis with killed *Treponema pallidum* and adjuvants, *J vener Dis Inform*, 28, 267

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Chapter 6

RESPONSE OF TREPONEMES TO DRUGS

It is not necessary to review here the voluminous literature that has accumulated on the effectiveness of penicillin in the treponemal diseases. Nor will we be concerned with a comparison of different kinds of preparations of penicillin in the various clinical syndromes encountered in man Suffice it to say that the discovery and development of penicillin has wrought a revolution in the treatment of these diseases; it is clear at the time of writing that penicillin G in one or another form exceeds all other therapeutic agents, both in respect of its effectiveness in eliminating treponemal infection in a human or animal host, and in its relative lack of toxicity for man

The latter quality is even more fortunate, since it does not apply equally to all mammalian species While penicillin virtually lacks toxicity, in the ordinary sense of the term, for man and most laboratory animals, including monkeys, rabbits, hamsters, rats and mice, it is rather highly toxic for guinea-pigs.[10, 20, 21] The basis for this species difference has not been determined It has been shown, however, that crystalline penicillin G in doses of 6 0 mg/kg body-weight of the guinea-pig is lethal to this species, while man readily tolerates doses as large as 100 000 000 units per day (about 1000 mg/kg body-weight) for many days [12] However, toxic reactions arising from hypersensitivity to penicillin or one of its components are not uncommon

Studies in this laboratory have dealt with the following four principle problems pertaining to the therapy of treponemal infections (1) the therapeutic effectiveness of different fractions of penicillin; (2) the effect of long-continued subcurative doses of penicillin during the incubation period of experimental syphilis; (3) the comparative therapeutic activity of other antibiotics in experimental syphilis; and (4) the comparative sensitivity of various strains and species of treponemes to penicillin.

During the first of these studies, a short *in vivo* method was developed for evaluating the comparative effectiveness of antibiotic agents in treponemal infection

62. Turner, T B & Nelson, R. A., jr. (1950) The relationship of treponemal immobilizing antibody to immunity in syphilis, *Trans Ass. Amer. Phys*, 63, 112

63. Turner, T B. et al, (1948) Protective antibodies in the serum of syphilitic patients, *Amer. J. Hyg.*, 48, 173

64 Waring, G W., jr & Fleming, W. L (1951) Further attempts to immunize rabbits with killed *Treponema pallidum*, *Amer. J. Syph*, 35, 568

65. Waring, G W., jr & Fleming, W L (1952) The effect of partial immunity on the dissemination of infection in experimental syphilis, *Amer. J. Syph*, 36, 368

66 Woods, A. C. & Chesney, A. M (1946) Relation of the eye to immunity in syphilis, with special reference to the pathogenesis of interstitial keratitis, *Amer J. Ophthal*, 29, 389

potent than fractions K or F. The data relative to fraction X were inadequate for comparative purposes, but suggested that this fraction was considerably less potent than fraction G

Because of the time-consuming and costly nature of the foregoing method of penicillin assay in laboratory animals, more rapid *in vivo* assay methods were sought. One of these methods was that developed by Eagle & Fleischman[6] in which rabbits were inoculated intradermally with 2000 *T. pallidum* and 4 days later were treated with varying amounts of one or another of the penicillin fractions. The least amount of drug required to abort the infection, as evidenced by failure to develop a syphiloma at the site of inoculation, was taken as an index of the therapeutic activity of the drug A somewhat similar procedure was developed by Rake, Dunham & Donovick.[19] These procedures require about 3-4 months from the beginning to the end of the assay.

Another method of assay, developed in our laboratory,[23] is based on the reduction of the numbers of *T pallidum* in cutaneous syphilomas following administration of the drug in question Rabbits are inoculated intracutaneously at multiple sites; when well-developed syphilomas are present, treponeme counts are made on 2 lesions, the drug is then administered in varying amounts to different groups of animals, and treponeme counts are made at appropriate intervals, usually every 24 hours, for the next few days The comparative effectiveness of a drug is determined by the rate of disappearance of treponemes In therapeutic doses, counts are usually reduced by at least 99% within 48 hours This assay requires only 48 hours, while the whole procedure, including the induction of suitable syphilomas in rabbits, requires less than 3 weeks.

A comparison of the results obtained by the various methods referred to above in the assay of different penicillin fractions is shown in Fig 10, which is adapted from a paper by Eagle & Fleischman[6] It will be noted that each of the *in vivo* methods gave essentially the same comparative information. It should also be noted that *in vitro* results obtained with the Reiter treponeme as the test organism[5] do not agree with the results obtained by *in vivo* methods using pathogenic treponemes

An interesting example of the application of basic knowledge to the study of a practical problem, yielding in turn additional basic knowledge, is seen in the study carried out in this laboratory and reported by Hollander, Turner & Nell[11] under the title " The effect of long continued subcurative doses of penicillin during the incubation period of experimental syphilis ". The prime objective of this study was to determine the extent to which long-continued subtherapeutic treatment with penicillin could modify or mask the course of infection with *T pallidum* in experimental animals Since the results of these experiments provide valuable confirmatory evidence of the sensitivity of *T pallidum* to penicillin, they will be briefly presented below

Methods of Assay

The assay of therapeutic agents potentially useful in the treponematoses is by no means easy, and relatively few studies of this nature have been made, although the day-to-day clinical use of some drugs has provided valuable information

In vitro tests for effectiveness have been practicable only since 1948, when Nelson (see Chapter 4) developed a method for maintaining the motility and virulence of pathogenic treponemes over a period of several days. *In vitro* assays require rigid controls, in order to detect non-specific effects on the motility of treponemes or on the deterioration of the drug in question, and a high degree of technical skill

The assay of drugs in infected animals is likewise a laborious and often a long-drawn-out affair. For example, the classical experiment on the effectiveness of different fractions of penicillin in experimental syphilis, conducted first under the auspices of the US Office of Scientific Research and Development, and later under the United States Public Health Service,[1] utilized approximately 1000 rabbits, and somewhat more than 9 months were required for a single comparative assay.

Clinical trials of drugs in man are even more difficult; few such large-scale tests have been attempted and fewer still have been brought to a clear-cut conclusion.[14] Nevertheless, the ultimate purpose in any therapeutic procedure is to obtain information concerning the probable effectiveness of the drug in man.

In vivo *methods*

In the case of the treponematoses, a bridge of sorts has laboriously been built between field experience on the one hand and laboratory results on the other through the large-scale studies on penicillin referred to above[1, 14] These studies were carried out in a number of clinics with a view to determining the effect of various dosage schedules in man The clinical study[14] showed that amorphous penicillin, consisting of a mixture of several penicillin fractions, gave results in early syphilis in man which were inferior to those obtained with a comparable dosage, in terms of Oxford units, of penicillin G

More or less concurrently an attempt was made to determine the approximate dose of various penicillin fractions which would cure 50% of rabbits infected with syphilis In the laboratory study, groups of rabbits were infected with *T. pallidum* and, after 6 weeks, were subjected to treatment with varying doses of penicillin fractions F, G, K or X. One hundred and twenty days after the termination of treatment, the lymph nodes of the treated rabbits were transferred to normal rabbits as a test of cure. The results of this study showed clearly that the G fraction of penicillin was more

the prolongation of the incubation period Other animals were given a second dose one week after the first dose, and showed a prolongation of the incubation period approximately twice that of the incubation period in animals receiving a single dose

TABLE XLIII INCUBATION PERIOD OF RABBITS INOCULATED INTRACUTANEOUSLY WITH 500 TREPONEMES AND GIVEN ONE SUBCURATIVE DOSE OF PENICILLIN 7 DAYS AFTER INOCULATION *

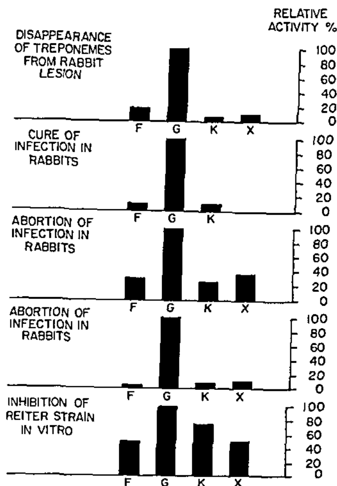
Experiment number	Penicillin dose (mg/kg body weight)	Incubation period in individual rabbits (days)	Mean incubation period (days)
I	Untreated	17 18 19, 20, 20	19
II	Untreated	17, 18 19 19 19	18
III	Untreated	17, 20 21, 23	20
I	0.004	16, 18 19 20, 26	20
I	0.02	17, 18, 18, 21, 21	19
I	0.1	22, 22 23 25	23
I	0.5	22, 23 23 23, 25	23
II	0.5	21, 22, 23 23, 23, 24	23
III	1.5	23, 28, 30, 31	28
II	2.5	23, 24, 24 25 30 30	26
III	3.0	30, 33 37	33

* From Hollander, Turner & Nell [11]

A procedure similar to that outlined can yield comparative data on the susceptibility of treponemal organisms to penicillin or other antibiotics, although it is a more time-consuming method than the simpler, but perhaps less precise, procedure developed by Turner, Cumberland & Li [23]

Data from the studies of Magnuson, Eagle & Fleischman [13] and Cumberland & Turner [4] on the multiplication time of treponemes (see Chapter 2), data on the effects of penicillin on experimental syphilis contributed by Eagle, Magnuson & Fleischman,[7] and Rake, Dunham & Donovick,[19] the *in vitro* results reported by Nell,[17] and the findings of the preliminary experiments mentioned above, suggested that a specific subcurative dose of penicillin kills a fairly constant proportion of the treponemes present Computations concerning the probable amount of penicillin required to maintain subclinical infection in rabbits for long periods were made on the basis of this information Obviously a nice balance was required between a dose which would cure the animals and one which would permit the infection to break through and produce clinically recognizable disease

FIG. 10. ACTIVITIES OF PENICILLINS F, G, K AND X AGAINST TREPONEMES



Relative activity against pathogenic treponemes in rabbits, measured by 4 different methods and against cultured Reiter treponemes

Reproduced from Eagle & Fleischman,[4] *by kind permission of the editors of the* Journal of Bacteriology

In preliminary experiments (Table XLIII) rabbits were inoculated intracutaneously at each of 4 sites on the back with 500 *T. pallidum*. Untreated controls quite regularly developed lesions after an incubation period of about 18 days. Penicillin G in aqueous solution was given intramuscularly in a single dose varying between 0.004 mg and 3.0 mg per kg of body-weight.

Animals receiving 0 1 *mg/kg or more* showed significant prolongation of the incubation period, the larger the dose the greater, in general, being

the prolongation of the incubation period. Other animals were given a second dose one week after the first dose, and showed a prolongation of the incubation period approximately twice that of the incubation period in animals receiving a single dose.

TABLE XLIII INCUBATION PERIOD OF RABBITS INOCULATED INTRACUTANEOUSLY WITH 500 TREPONEMES AND GIVEN ONE SUBCURATIVE DOSE OF PENICILLIN 7 DAYS AFTER INOCULATION *

Experiment number	Penicillin dose (mg kg body-weight)	Incubation period in individual rabbits (days)	Mean incubation period (days)
I	Untreated	17 18, 19, 20, 20	19
II	Untreated	17 18 19, 19 19	18
III	Untreated	17, 20 21, 23	20
I	0.004	16, 18 19, 20, 26	20
I	0.02	17, 18 18 21, 21	19
I	0.1	22, 22 23 25	23
I	0.5	22 23, 23 23, 25	23
II	0.5	21, 22, 23 23 23, 24	23
III	1.5	23 28 30 31	28
II	2.5	23, 24 24 25, 30, 30	26
III	3.0	30 33 37	33

* From Hollander, Turner & Nell[11]

A procedure similar to that outlined can yield comparative data on the susceptibility of treponemal organisms to penicillin or other antibiotics, although it is a more time-consuming method than the simpler, but perhaps less precise, procedure developed by Turner, Cumberland & Li[23]

Data from the studies of Magnuson, Eagle & Fleischman[13] and Cumberland & Turner[4] on the multiplication time of treponemes (see Chapter 2), data on the effects of penicillin on experimental syphilis contributed by Eagle, Magnuson & Fleischman,[7] and Rake, Dunham & Donovick,[19] the *in vitro* results reported by Nell,[17] and the findings of the preliminary experiments mentioned above, suggested that a specific subcurative dose of penicillin kills a fairly constant proportion of the treponemes present. Computations concerning the probable amount of penicillin required to maintain subclinical infection in rabbits for long periods were made on the basis of this information. Obviously a nice balance was required between a dose which would cure the animals and one which would permit the infection to break through and produce clinically recognizable disease

On the basis of these computations, 46 rabbits were inoculated in a similar manner with 500 *T. pallidum* at each site (Table XLIV). Four animals were untreated. Half the remaining animals were given 2 0 mg/kg in weekly injections and half were given 4 0 mg/kg In each group, 2 animals were given 2 treatments, 2 were given 4 treatments, 2 were given 6 treatments, and so on up to 20 weekly treatments.

TABLE XLIV INCUBATION PERIOD OF RABBITS INOCULATED INTRACUTANEOUSLY WITH 500 TREPONEMES AND GIVEN SUBCURATIVE DOSES OF PENICILLIN AT WEEKLY INTERVALS DURING THE INCUBATION PERIOD *

Rabbit number	Treatment dosage (mg/kg)	Incubation period (days)	
		theoretical	observed
30-77	2× 2 wks	31	34
30-94	2× 2 wks	31	31
30 80	2× 4 wks	45	49
30 91	2× 4 wks	45	43
30-83	2× 6 wks	59	66
30 86	2× 8 wks	73	87
30-82	2×10 wks	87	98
30-78	2×16 wks	129	140
30 81	2×18 wks	144	146
30-93	2×18 wks	144	144
30-84	2×20 wks	158	167
30 96	2×20 wks	158	156
30 98	4× 2 wks	31	31
31-15	4× 2 wks	31	30
31-12	4× 4 wks	45	49
31-09	4× 6 wks	59	(106) a
31-06	4× 8 wks	73	85
31-00	4×12 wks	101	101
30 97	4×14 wks	115	121

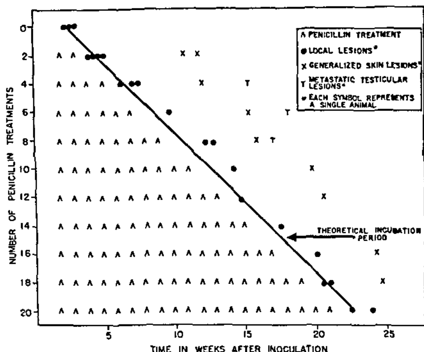
* From Hollander, Turner & Nell [44]

a No lesions appeared at the inoculation sites in this animal Generalized lesions were noted on the 106th day

It was calculated that upon the termination of the weekly injections of supposedly subcurative doses of penicillin syphilitic lesions would develop at the site of inoculation approximately 7 days after the last injection. The usual course of syphilitic infection appears to be determined by a

regular logarithmic increment of treponemes which continues until sufficient numbers are present to produce a lesion, and a single dose of penicillin seems to destroy a percentage of the organisms present in proportion to the size of the dose. The potency of a single subcurative dose given at any time during the incubation period can therefore be measured by the prolongation of the incubation period which it induces.

FIG. 11. INFLUENCE OF REPEATED SUBCURATIVE DOSES OF PENICILLIN ON THE INCUBATION PERIOD OF EXPERIMENTAL SYPHILIS



Rabbits inoculated intracutaneously with 500 treponemes were given either 2 mg or 4 mg of penicillin G per kg of body-weight at the indicated weekly intervals. More or less typical syphilitic lesions occurred in many of the animals shortly after the last treatment. Details of this experiment are to be found in the text.

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Pursuing this line of reasoning, since the destruction of 50% of the organisms at any one time is equivalent to the deletion of one generation, the resultant delay in incubation period will be about 30-33 hours, a period which is believed to be the division time of this organism.[4, 13] Similarly,

On the basis of these computations, 46 rabbits were inoculated in a similar manner with 500 *T. pallidum* at each site (Table XLIV) Four animals were untreated. Half the remaining animals were given 2.0 mg/kg in weekly injections and half were given 4.0 mg/kg. In each group, 2 animals were given 2 treatments, 2 were given 4 treatments, 2 were given 6 treatments, and so on up to 20 weekly treatments.

TABLE XLIV INCUBATION PERIOD OF RABBITS INOCULATED INTRACUTANEOUSLY WITH 500 TREPONEMES AND GIVEN SUBCURATIVE DOSES OF PENICILLIN AT WEEKLY INTERVALS DURING THE INCUBATION PERIOD *

Rabbit number	Treatment dosage (mg/kg)	Incubation period (days)	
		theoretical	observed
30-77	2× 2 wks	31	34
30 94	2× 2 wks	31	31
30-80	2× 4 wks	45	48
30 91	2× 4 wks	45	43
30-83	2× 6 wks	59	66
30 86	2× 8 wks	73	87
30-82	2×10 wks	87	98
30-78	2×16 wks	129	140
30 81	2×18 wks	144	146
30 93	2×18 wks	144	144
30-84	2×20 wks	158	167
30 96	2×20 wks	158	156
30-98	4× 2 wks	31	31
31 15	4× 2 wks	31	30
31-12	4× 4 wks	45	49
31-09	4× 6 wks	59	(106) *a*
31-06	4× 8 wks	73	85
31-03	4×12 wks	101	101
30 97	4×14 wks	115	121

* From Hollander, Turner & Nell [11]

a No lesions appeared at the inoculation sites in this animal Generalized lesions were noted on the 106th day

It was calculated that upon the termination of the weekly injections of supposedly subcurative doses of penicillin syphilitic lesions would develop at the site of inoculation approximately 7 days after the last injection The usual course of syphilitic infection appears to be determined by a

this laboratory Anaerobiosis is ensured by layering paraffin oil containing 0.1% of 2, 6-di-tert-butyl *p*-cresol, an oil soluble antioxidant, to a height of 1.5 cm. over the aqueous phase. Samples of treponemes for darkfield examination were removed with sterile capillary pipettes.

In each penicillin sensitivity test the following controls were included: (*a*) survival control to determine the proportion of treponemes remaining motile in the absence of an antibiotic, ordinarily 80% to 100%; and (*b*) antibiotic stability control on the penicillin or other antibiotic mixture, accomplished by including one or more tubes containing a *Staphylococcus aureus* of known susceptibility to the drug

Penicillin sensitivity was determined by exposing treponeme suspensions to graded doses of the antibiotic for 18 hours at 35°C, and then comparing the proportion of motile treponemes present in the test mixtures with the proportion motile in the control mixture. Under high dry-magnification 50 or 100 successive treponemes in consecutive fields were examined, and the number of motile organisms recorded The antibiotic concentration required to immobilize 50% of the observed organisms was taken as an index of the susceptibility of treponemes to penicillin, as follows

$$\%\ \text{motile in test mixture} = \frac{\%\ \text{motility in test mixture}}{\%\ \text{motility in control}} \times 100$$

Factors affecting in vitro *assay of penicillin sensitivity*

It should be evident from the foregoing description of the technique of assay of the penicillin sensitivity of pathogenic treponemes that the method is by no means simple There are ever-present problems with respect to the avoidance of contamination in working with organisms derived from animal sources Moreover, while survival of treponemes in the control suspensions is usually good, at times it was unaccountably poor.

Nell[17] also examined other factors which might play a role in the *in vitro* action of penicillin on treponemes·

1. Concentration of treponemes in the test suspension. Within the range of concentrations of treponemes used (5×10^{5} to 30×10^{6}) the activity of penicillin was apparently not significantly influenced by the numbers of treponemes present.

2 Temperature of incubation. Sensitivity tests with different dilutions of penicillin were made simultaneously at 25°C, 30°C and 35°C, and motility counts were made at 12, 18 and 24 hours (Fig 12) At 25°C, a concentration of 0 005 μg per ml penicillin had no demonstrable effect after 18 hours, at 30°C, 50% were immobilized; while at 35°C, 87% were immobilized Comparable results were obtained with higher concentrations of penicillin These results indicate that in the range between 25° and 35°C penicillin can be active against treponemes, but that the rate at which

destruction of 90% of the organisms will cause a prolongation of about 4½ days; 99%, a delay of 9 days; 99.9%, a delay of 13½ days, and so forth.

Of 20 surviving animals that received weekly injections of 4 mg/kg, 13 were cured (as determined by lymph-node transfer), and the remaining 7 had complete suppression of syphilitic lesions until after the termination of treatment.

Of 20 surviving rabbits receiving the smaller weekly dose of penicillin, 4 were cured, 12 had complete suppression of lesions until after the termination of treatment, and 7 had partial suppression of lesions for 3 to 9 weeks, among these latter animals the lesions were usually small and non-progressive.

In Table XLIV and Figure 11 are shown the theoretical and observed incubation periods for these animals In general, the observed figures are in accord with the theoretical figures The implications of this study in terms of the production of immunity were noted in Chapter 5.

In vitro *methods*

The most extensive *in vitro* assays using pathogenic treponemes as the test organism thus far reported are those carried out by Nell [17] in this laboratory. The method employed was briefly as follows.

Animals were infected by inoculation of large numbers of treponemes into each testis Within a few days after development of orchitis, the testes were removed, and treponemes were extracted in a manner similar to that described for the TPI test (see Chapter 5). The treponeme suspension was centrifuged lightly in order to remove red blood cells, spermatozoa, and gross tissue debris. *The treponeme concentration in the final suspension* commonly varied between 5×10^6 and 10×10^6 per ml

To avoid variation due to errors of dilution a stock solution of penicillin or other drugs to be tested was prepared and maintained in the frozen state at -20° C. The stock solution of crystalline penicillin G was prepared in saline in a final concentration of 2.65 μg per ml and final dilutions to be used in the test proper were prepared in the same medium that was used for extraction of treponemes.

Parallel dilutions were made up to a total volume of 0.5 ml. These dilutions contained 4 times the concentration of penicillin needed in the final test suspension, the volume of which was 2 ml. To 0.5 ml of penicillin dilution contained in sterile test tubes measuring 13 mm × 100 mm was added 1.5 ml of the treponeme suspension The tubes were well shaken and incubated in a Brewer anaerobic jar under an atmosphere of 5% carbon dioxide and 95% nitrogen at 35°C for 18 hours

In all experiments necessitating multiple readings from the same test mixture, an oil barrier to exclude oxygen from the test mixtures was used instead of the anaerobic jar. This technique was designed by Weber [25] in

In vitro *sensitivity of the Nichols strain of* T. pallidum

In vitro assays with the Nichols strain of *T. pallidum* gave 50% immobilization in 10 different assays at concentrations varying between 0 0011 μg and 0.0031 μg, the mean figure being 0 002 μg. These figures are of the same order of magnitude as the serum concentration found by Eagle, Magnuson & Fleischman [7] to be necessary to cause the disappearance of treponemes from syphilitic lesions of experimental animals

Comparative results with other strains of treponemes will be presented in Chapter 9

Comparative Effect of Various Antibiotics in Experimental Syphilis

There is no antibiotic now available that approaches the therapeutic efficiency of penicillin in the treponematoses. Clinical studies indicate, however, that a number of the newer antibiotics have some therapeutic value in syphilis, and presumably in the other treponematoses Since there is always the possibility that some strains of treponemes may eventually develop resistance to penicillin, and since an increasing number of individuals are becoming hypersensitive to penicillin, it is desirable to have information on the probable range of effectiveness of other antibiotics.

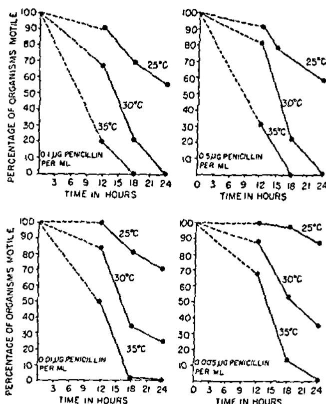
Many of the following comparative observations with penicillin, Terramycin, Chloromycetin, streptomycin, erythromycin, Magnamycin, iodides and gold have been reported by Turner & Schaeffer.[24] The method of study was essentially that developed in this laboratory,[23] and consisted in brief of the induction of syphilomas by intracutaneous inoculation of rabbits with *T. pallidum*, and determination of treponeme counts on these lesions both before and after the administration of the antibiotic to be tested.

The Nichols strain of *T. pallidum* was used throughout Rabbits' testes showing an early syphilitic orchitis were emulsified in 10% rabbit serum in physiologic saline, the emulsion centrifuged to sediment gross tissue particles, and the supernatant diluted so that 0 1 ml contained approximately 50 000 treponemes This amount was inoculated intracutaneously in each of 8 sites on the clipped backs of normal male rabbits

Syphilitic lesions usually develop after an incubation period of about 7 days, and within another 3-5 days typical syphilomas of uniform size are commonly present in all inoculated animals

Treponeme counts are made by grasping the base of a lesion with a hemostat in order to fix the lesion and at the same time effect hemostasis. The top of the syphilitic nodule is then sliced off with a sharp razor-blade and approximately 0 05 ml of serum collected on each of 2 slides. The number of treponemes in 100 oil immersion fields is then counted under the darkfield for each preparation and the count expressed as the number per

FIG. 12 INFLUENCE OF TEMPERATURE ON THE ACTIVITY OF PENICILLIN "IN VITRO"



Percentage survival at indicated time, temperature and penicillin concentration
Reproduced from Nell,[11] by kind permission of the editors of the American Journal of Syphilis, Gonorrhea and Venereal Diseases

treponemes are immobilized by a given concentration varies directly with the temperature.

3. Rate of treponemicidal action. In general the rate of immobilization of treponemes by penicillin is a function of the drug concentration and the temperature. However, a concentration greater than 0.001 µg per ml was required before any effect on the motility of treponemes became demonstrable within a 24-hour period. At the other end of the scale, the maximum rate of immobilization was observed with a concentration of about 0.1 µg per ml. Concentrations from 0.1 µg per ml to 50 µg per ml produced essentially the same results, but always after 4 hours during which no immobilization was noted.

TABLE XLV EFFECTS OF VARIOUS ANTIBIOTICS AND GOLD ON *SPIROCHETE COUNTS IN EXPERIMENTAL SYPHILIS* *

Antibiotic	*Number* of rabbits	Dose (mg kg body weight)	Route of injection	Rabbits showing reduction of counts to indicated number [a]		
				100 200	50 200	10 200
Aureomycin	4	50	Intravenous	4	3	3
"	2	25	"	1	1	1
"	2	10	"	0	0	0
Terramycin	3	50	Intravenous	3	3	2
"	3	25	"	1	1	0
"	2	25	Intramuscular	2	2	2
"	2	10	Intravenous	1	0	0
"	2	10	Intramuscular	0	0	0
Chloromycetin	2	50	Intramuscular	1	0	0
"	2	25	"	0	0	0
Streptomycin	2	50	Intramuscular	2	1	0
"	2	25	"	0	0	0
Erythromycin	5	300	Intramuscular	5	5	5
"	3	50-60		3	3	3
"	2	25		2	2	2
"	5	10	"	4	4	1
"	4	5	"	2	2	0
	5	1	"	1	1	0
Magnamycin	2	50	Intramuscular	2	2	2
"	2	10	"	2	2	2
"	2	5	"	2	2	1
	6	2	"	6	4	2
	4	0 5		4	3	1
"	2	0 25	"	2	2	0
"	2	0 15		2	2	0
"	4	0 1	"	1	1	0
Penicillin	3	0 25	Intramuscular	3	3	3
"	2	0 2	"	2	2	2
	2	0 05	"	1	0	0
Gold	2	10 [b]	Intramuscular	2	2	2
	2	2	"	0	0	0

* From Turner & Schaefer [14]

[a] Numerator = number of treponemes, denominator = *number of fields*

[b] In terms of metal only

200 fields. Most animals under these circumstances will show a pre-treatment count of 600 to 1200 Animals with counts below 200 (only exceptionally encountered) are not used.

The total dosage of the antibiotic or other drug to be tested expressed in terms of milligrams of drug per kilogram of body-weight of the animal, was commonly administered in 2 equal dosages 6 hours apart. Drugs were given either intramuscularly into the thigh muscles, or intravenously into the marginal ear vein Post-treatment counts were made as described on each day after treatment through the 6th or 7th day, the counts being made each time on a previously unused syphiloma.

Results of in vivo *assays*

Penicillin. Turner, Cumberland & Li [23] determined that crystalline penicillin G when given in doses of 0.25 mg/kg or greater will almost regularly bring about, within 24 hours, a reduction in the treponeme count to 10 or fewer per 200 fields, even when the pre-treatment count is very high, i.e., of the order of 1000 to 1500 treponemes per 200 fields. The low count may persist for another 24-48 hours, but often after this period the count will begin to rise It is estimated that 0 11 mg/kg is the dosage which will reduce the count to 10 treponemes or fewer in 24 hours in half of the rabbits

It should be noted that while in the earlier studies in this laboratory [23] penicillin was divided into 3 equal dosages at 2-hour intervals, in the more recent experiments [24] penicillin was given in 2 equal doses 6 hours apart. It would appear that the later results, given in Table XLV are roughly comparable to those obtained in the earlier studies. The preparation used in these experiments was crystalline penicillin G in aqueous solution. Intramuscular injection was used routinely. Maximum reduction in counts was always observed within 24-48 hours after administration of the drug.

Aureomycin. Aureomycin is customarily given orally but may be administered parenterally. The preparation used in these experiments was Aureomycin Hydrochloride Crystalline produced by Lederle Laboratories Division for intravenous use The crystalline material was dissolved in physiologic saline so that 1 ml contained 10 mg Aureomycin. Administration was by the intravenous route.

The results are shown in Table XLV. Both 50 and 25 mg/kg had demonstrable therapeutic effect, while 10 mg/kg did not. Maximum reduction in counts was observed 3-5 days after treatment. These dosage levels, however, did not give as good results as did 0.25 mg/kg penicillin.

Terramycin. Terramycin is customarily administered orally, but preparations for both intravenous and intramuscular use are available. Two products were tested· Crystalline Terramycin Hydrochloride Intravenous prepared by Chas. Pfizer & Co., Inc., and a preparation designed for intra-

Magnamycin Magnamycin (carbomycin) is prepared by Chas. Pfizer & Co., Inc. It is customarily administered orally, but a preparation in powder form designed for intramuscular administration was supplied by the manufacturers. The material was dissolved in distilled water so that the individual dose (i.e., one-half the total dose) would be contained in 1-2 ml of solution

The results of intramuscular administration of various dosages are given in Table XLV. The activity against *T. pallidum* begins to approach that of penicillin but falls considerably short of equaling it. The lowest counts were observed 3-5 days after treatment.

Effect of miscellaneous drugs

Gold salts. The preparation tested was a solution of sodium gold thiomalate, which contains 50% gold and is marketed by Merck and Company, under the name Myochrysine This drug is used primarily in the treatment of the active stages of rheumatoid arthritis The tests for treponemicidal activity were made with total doses of 10 mg/kg and 2 mg/kg of heavy metal (i e , 20 mg/kg and 4 mg/kg, respectively, of Myochrysine solution) administered intramuscularly in 2 equal doses 6 hours apart

It will be noted (Table XLV) that, in total doses of 10 mg/kg, gold was effective in reducing the spirochete count to a low level The lowest counts were observed 3-7 days after treatment. With doses of 2 mg/kg little effect was noted

Sodium iodide Four animals with typical cutaneous syphilomas were given sodium iodide solution intravenously in total doses of 200 mg/kg administered in equal amounts 24 hours apart in a 10% solution. In each animal a moderate reduction in spirochete count was noted within 24 hours after the initial dose, but the drug had no striking treponemicidal effect in the doses used

Significance of results

All the antibiotics tested in these studies showed some treponemicidal activity It should be re-emphasized that not only is the experimental method highly artificial with respect to the treatment of syphilitic infection in human beings, but also the drugs themselves were often not administered by the optimum route or at the optimum time interval between doses. Moreover, no observations were made on the effectiveness of any of these antibiotics when administered over a longer period of time.

Nevertheless, it is probable that the results obtained by this short *in vivo* assay method provide reasonably reliable evidence on the relative treponemicidal activity of the various antibiotics and other drugs tested Even this limited survey entailed the use of a large number of rabbits and many man-hours of professional application in making spirochete counts *In vitro*

muscular use, which was supplied by the same company. For intravenous administration the crystalline material was dissolved in physiologic saline so that 1 ml contained 10 mg of drug For intramuscular administration it was dissolved in distilled water.

The results when total dosages of 50, 25, and 10 mg/kg were given are shown in Table XLV The antitreponemal effect was similar to that of Aureomycin and much less than that of penicillin in dosages of 0 25 mg/kg Maximum reduction in counts was noted 3-5 days after treatment.

Chloromycetin Chloromycetin (chloramphenicol) is customarily given by mouth, but preparations for intravenous administration are available The product tested was Chloromycetin Solution prepared by Parke, Davis and Company Two ml of solution contained 500 mg of chloromycetin in a 50% aqueous solution of N, N-dimethylacetamide; for intravenous administration the preparation was either used in this strength, or further diluted in physiologic saline so that each ml contained 50 mg.

The results of administering total dosages of 50 and 25 mg/kg intramuscularly are shown in Table XLV. While a reduction in the treponeme count from the pre-treatment level was noted, this was not as great as with either Aureomycin or Terramycin Maximum reduction in counts occurred 3-5 days after treatment

Streptomycin. The product tested was Streptomycin Sulfate prepared by E. R Squibb & Sons and supplied as a sterile powder readily soluble in aqueous solution for intramuscular injection The powder was diluted in physiologic saline so that 1 ml of solution contained 100 mg of drug

The results obtained with 50 and 25 mg/kg intramuscularly are shown in Table XLV Again, while a reduction in treponemes from the pre-treatment level was noted, the reduction was not as great as that observed with Aureomycin or Terramycin The lowest counts occurred 3-5 days after treatment

Erythromycin Erythromycin can be administered orally or intramuscularly. The product was prepared by Eli Lilly and Co. and is known as Ilotycin. The initial batch of material, employed in tests with the higher dosage levels (10 mg/kg and up), was used in a 5% aqueous suspension A second lot, supplied to us in a stable solution, was used for tests with the lower dosages. In each instance administration was by the intramuscular route.

The results obtained with various dosages are shown in Table XLV. It is evident that this antibiotic shows a higher degree of activity than Aureomycin and Terramycin but still does not equal the antitreponemal activity of penicillin. It would appear also from these limited data that erythromycin is not quite as active against *T. pallidum* as Magnamycin (see below) The maximum reduction in counts was observed 3-5 days after treatment

It has become clear, from studies on other micro-organisms, that penicillin is active principally on dividing organisms rather than on those that are in a resting phase. Some of the observations on treponemes are consistent with the notion that, in this group of organisms too, penicillin is active mainly on rapidly dividing treponemes.

It has been said that long forms of treponemes are proportionately more abundant late in the course of treponemal infection, while short forms predominate early. This suggests that a predominance of short forms is indicative of a phase in which the treponemes are rapidly dividing

Frazier & Frieden [8] reported that within 90 minutes after the injection in a patient of 20 000 units of penicillin the ratio of long forms of *T. pallidum* to short forms increased from 1 : 22 to 1 5. In a second case which received 40 000 units the ratio changed after 6 hours from 1 . 10 to 1 . 3. Observations were also made by Morton & Ford [15] on 3 patients with lesions of early syphilis. Penicillin was given in several doses of 40 000 units every 2 hours, *and darkfield examinations were made as long as treponemes were demons*trated by darkfield. In all 3 patients treponemes disappeared so rapidly from lesions that few measurements could be made, but these did not indicate a significant shift to longer forms.

In the case of the experimental disease, we have had extensive experience in the observation of treponemes from lesions following penicillin therapy. The most striking phenomenon is the rapid reduction in the number of treponemes of all lengths. Following an effective dose of aqueous penicillin administered intramuscularly, the reduction begins to take place in about 4 hours and is well advanced by 6 hours Long forms of treponemes do become proportionately more numerous, but they are by no means exclusively present.

The mechanism by which treponemes are cleared from syphilitic lesions is obscure. The rapidity with which they disappear and the absence of distorted forms suggest that lysis occurs *in vivo* On the other hand it cannot be certain that phagocytosis does not play a significant role. In cortisone-treated animals, although the situation is different, the underlying mechanism is no less obscure. Following cortisone treatment, syphilitic lesions commonly show a great increase both in the amount of mucoid material and in the number of treponemes. Upon the administration of penicillin the treponemes in these lesions appear to be killed, but the rate of disappearance of the dead treponemes is greatly decreased This observation suggests that phagocytosis, which is inhibited under these conditions by the relative absence of cellular elements, and possibly by mechanical interference from the mucoid material, is the underlying mechanism by which lesions are ordinarily cleared of treponemes, rather than lysis

A number of studies have been made on the effects of penicillin on the Reiter spirochete, but, as mentioned previously, there is a question as to the extent to which those observations can be interpreted in terms of pathogenic

assay methods appear to offer no substantial advantages over the *in vivo* methods employed here, for they are scarcely less time-consuming; *T. pallidum* is notoriously difficult to work with *in vitro*; and the results when obtained are perhaps less meaningful in terms of actual therapy.

The results of this study are in accord with a number of clinical observations in which Aureomycin, Chloromycetin, Terramycin and streptomycin have been used in the treatment of patients with early syphilis. In all instances some treponemicidal action was demonstrated, but without exception it was less striking than that obtained with penicillin. Similar clinical trials have also been made in patients with yaws and pinta. (The relevant literature is cited by Turner & Schaeffer [21])

No reports of clinical trials of erythromycin and Magnamycin in early syphilis have come to the authors' attention. Gold is known to have some antisyphilitic activity [21] similar perhaps to that of certain other heavy metals, notably mercury and bismuth. Studies on the iodides have shed no light on the well-known therapeutic effect of potassium iodide in patients with cutaneous gummata, since only feeble treponemicidal effect was observed with large doses Kolmer and his associates (cited by Turner & Schaeffer [21]) have reported that, while the iodides alone have little therapeutic effect in experimental syphilis, this drug does seem to act synergistically or at least additively with penicillin, with combined penicillin and mercury succinimide, and with the arsenicals, although the data are limited and the point cannot be regarded as definitely proved.

The data obtained in these studies, while limited, do permit a rough approximation of the comparative treponemicidal properties of the various antibiotics tested Within the obvious limitations of the method and of the number of observations, the following order of effectiveness, beginning with the most active, is suggested (1) penicillin, (2) Magnamycin and erythromycin, (3) Terramycin and Aureomycin, (4) Chloromycetin and streptomycin With respect to treponemicidal potency, penicillin, of course, remains in a class by itself

By the assay method used, large doses of most of these antibiotics were required to produce a measurable antitreponemal effect. Nevertheless it seems probable that, over the years, the widespread use of even small amounts of many of these antibiotics in miscellaneous infections, both minor and severe, may exert a substantial, preventive and therapeutic effect on syphilis and related treponemal diseases.

Mode of Action of Penicillin on Treponemes

The unique susceptibility of most strains of treponemes to penicillin has been adequately demonstrated through extensive clinical experience. Virtually nothing, however, is known concerning the mode of action of penicillin on treponemes.

In our laboratory the senior author in association with Dr Huang-Ying Li attempted to induce penicillin resistance by serial passages of the Nichols strain of *T. pallidum* in animals which had received subcurative doses of penicillin. After one year, during which the strain was carried through approximately 8 passages under these conditions, there was no evidence of increased resistance to penicillin

More recently Probey [19] has attempted to induce penicillin resistance through the administration of a single massive dose of penicillin to each of 3 successive passage rabbits Therapeutic assays with this experimental strain and its normal counterpart failed to reveal any evidence of penicillin resistance

The experiments of Hollander, Turner & Nell [11] are pertinent in this connexion Rabbits infected with the Nichols strain of *T pallidum* were given weekly subcurative doses of penicillin for periods up to 20 weeks (see Table XLIV, page 174) Upon withdrawal of penicillin at the end of this time, the evolution of the syphilitic infection did not deviate essentially from its usual pattern, and the strain of treponemes when tested by the procedure of Turner, Cumberland & Li [23] showed no divergence from the usual pattern of penicillin susceptibility

It is too early, however, to state with confidence that resistant strains of treponemes will not emerge Penicillin has been used widely in only a few countries for as long as 10 years, while in many other areas populations have been " penicillinized " for much shorter periods of time Some surveillance of freshly isolated strains from various parts of the world should be maintained in order to detect variations, if any, toward the development of resistance to penicillin In view of the virtually complete reliance on penicillin as the therapeutic foundation of world-wide treponematosis control this would seem to be a reasonable precaution

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4 [illegible]

5 [illegible]

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treponemes Tung & Frazier [22] found that in cultures of Reiter treponemes, to which sublethal concentrations of penicillin had been added, elongated forms appeared after 24 hours Morton & Oskay [16] likewise noted elongated forms of the Reiter spirochete in cultures to which sublethal concentrations of penicillin had been added It should be noted that, in these studies with culture treponemes, death but not lysis of the organisms occurred upon exposure to penicillin

Combined Penicillin and Cortisone

It seems evident from the foregoing studies as well as from much circumstantial evidence that penicillin acts directly on the treponeme rather than indirectly through some host mechanism It seems clear, too, that this antibiotic is equally effective *in cortisone-treated animals*, and probably in cortisone-treated patients as well

Since cortisone treatment suppresses many types of acute tissue reaction, there is a rational basis for the use of this drug simultaneously with penicillin in patients in whom a Herxheimer reaction is feared or in individuals known to be hypersensitive to penicillin.[3, 9] Indeed the proposal has been made that cortisone should be administered routinely at the beginning of penicillin therapy for syphilis [9] On the basis of knowledge concerning the histological changes induced in syphilitic lesions by cortisone, as described in Chapter 3, it might be reasoned that cortisone, by suppressing the indurated type of tissue reaction, would permit more effective penetration of the antibiotic, although we are unaware of any direct evidence bearing on this point

From an altogether different standpoint, the rebound phenomenon observed in experimental syphilis following the withdrawal of cortisone (see Chapter 3) can be eliminated by the timely administration of penicillin. While this phenomenon probably presents no serious problem in clinical syphilology, the controlling action of penicillin would doubtless be equally evident in man Thus it might be stated that in the treponematoses of man cortisone may be used to prevent undesirable reactions from penicillin, and penicillin to prevent undesirable reactions from cortisone.

The Question of Penicillin Resistance

In view of the rapid development of penicillin resistance in some microorganisms, students of the treponematoses have been on the alert for evidence pointing to the development of penicillin resistance on the part of pathogenic treponemes Happily, no credible evidence has come to our notice suggesting such a contingency and in a thorough review of the literature, in 1950, Beerman and his associates [2] came to the same conclusion

In our laboratory the senior author in association with Dr Huang-Ying Li attempted to induce penicillin resistance by serial passages of the Nichols strain of *T. pallidum* in animals which had received subcurative doses of penicillin After one year, during which the strain was carried through approximately 8 passages under these conditions, there was no evidence of increased resistance to penicillin

More recently Probey [18] has attempted to induce penicillin resistance through the administration of a single massive dose of penicillin to each of 3 successive passage rabbits Therapeutic assays with this experimental strain and its normal counterpart failed to reveal any evidence of penicillin resistance.

The experiments of Hollander, Turner & Nell [11] are pertinent in this connexion. Rabbits infected with the Nichols strain of *T. pallidum* were given weekly subcurative doses of penicillin for periods up to 20 weeks (see Table XLIV, page 174). Upon withdrawal of penicillin at the end of this time, the evolution of the syphilitic infection did not deviate essentially from its usual pattern, and the strain of treponemes when tested by the procedure of Turner, Cumberland & Li [23] showed no divergence from the usual pattern of penicillin susceptibility

It is too early, however, to state with confidence that resistant strains of treponemes will not emerge Penicillin has been used widely in only a few countries for as long as 10 years, while in many other areas populations have been " penicillinized " for much shorter periods of time Some surveillance of freshly isolated strains from various parts of the world should be maintained in order to detect variations, if any, toward the development of resistance to penicillin. In view of the virtually complete reliance on penicillin as the therapeutic foundation of world-wide treponematosis control this would seem to be a reasonable precaution

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Part II

COMPARATIVE STUDY OF STRAINS OF TREPONEMES

General considerations

We shall now come to grips with one of the major problems to which the activities of this laboratory have been directed, namely, the relationship between strains and species of treponemes It is a problem which is so wide in extent and reaches so far back in time that few individuals can from first-hand knowledge speak about it with high scientific precision.

But from the vantage point of the World Health Organization it is now possible to make a world-wide study of the treponematoses; to learn the true geographical distribution of this group of diseases, to define their clinical and epidemiological patterns in a common scientific language; to seek the basis for any differences in these patterns, and, finally, to extract the essence of the knowledge thus acquired for the benefit, it is hoped, of mankind in many lands.

The studies to be presented here form only one segment of the activities directed to this high purpose, but it is an important segment With the assistance of many individuals we have collected strains of treponemes from patients afflicted with one or another of the treponematoses, in each instance the patient from whom the strain was obtained was regarded as presenting a clinical and epidemiological picture typical of the predominant pattern in the particular area All these strains have been brought to a single laboratory, propagated in animals maintained under the same environmental conditions, and studied by the same small group of investigators.

In conducting these comparative studies we have been constantly seeking to move from a qualitative to a quantitative level of investigation In this case it has not been easy Our particular difficulty lies in the fact that in many instances yardsticks which will truly measure a basic biological property are not readily found

Yet, while we shall not hesitate to record pertinent qualitative observations, our purpose is to attempt to characterize the behavior of these various strains of treponemes by indices which will remain valid and usable *from one laboratory to another.*

The studies in this laboratory, carried out under the guidance of the World Health Organization, have coincided with a number of clinical and epidemiological studies which are designed to give a clear picture of the syndromes of the treponematosis group. We shall be only indirectly concerned with these, but in the event that distinctive features are observed

among these different syndromes, we may be able to contribute to the solution of the inevitable question as to what may be the basis for those differences.

As mentioned previously, for the purposes of this comparative approach we have adopted the device of designating strains according to the geographical area in which they were isolated rather than by the clinical syndrome of the case from which they were derived. Thus, the strains may be catalogued without reference to pre-conceived notions as to what characteristics they ought to show Later, of course, it will be necessary to review all these data in relation to the treponemal syndromes mentioned above.

The comparative study of strains of treponemes presented here will encompass three principal kinds of data, namely: (*a*) characteristics of the disease in laboratory animals; (*b*) immunological relationships; and (*c*) behavior in response to antibiotics.

Chapter 7

COMPARATIVE CHARACTERISTICS OF THE EXPERIMENTAL DISEASE INVOKED BY VARIOUS STRAINS OF TREPONEMES

Comparative data are available principally on the disease picture in rabbits and to a less extent in hamsters, useful data are not available for other laboratory animals

The Disease Picture in Rabbits

The general characteristics of experimental treponemal infection in the rabbit have been presented in Chapter 2 In that section as well as in the published work of Pearce & Brown,[10] Matsumoto [8] and Turner & Chesney,[11] it has been pointed out that in general strains of treponemes obtained from patients with syphilis tend to differ from those obtained from patients with yaws The four features most commonly observed in experimental yaws infection that seem to differentiate it from experimental syphilis in the rabbit are (*a*) the relative absence of induration in the initial testicular lesions, (*b*) small focal lesions in or immediately beneath the visceral tunic of the testis, giving rise to a picture referred to as granular periorchitis, (*c*) the relative absence of induration in initial lesions of the skin, and (*d*) the relative scarcity of generalized lesions

During the course of the studies in this laboratory we have had the opportunity to look for three of these four characteristics in all of the strains studied, we have not made adequate observation in respect of generalized lesions because of the much longer period required to accumulate reliable data on each animal.

Method of comparison

All infected rabbits were customarily examined twice a week and usually more often during the period of most active evolution of lesions Examination included palpation of inoculated testes and recording of abnormalities such as enlargement, or induration, or the presence of a granular periorchitis

In each animal inoculated intratesticularly the lesion has been classified according to its extent at its maximum development, by assigning a numerical grade of 1, 2, 3 or 4. Ordinarily, inoculations were made in only one testis, in which case the opposite testis served as a standard of reference. The classification is based roughly on the following criteria:

Grade 1. Lesion represents only slight deviation from normal in either size or firmness.

Grade 2 Slight changes in the body of the testis as in grade 1, with enlargement and induration of the head of the epididymis in addition.

Grade 3. Testis enlarged with indurated nodules in the body of the organ.

Grade 4. Marked enlargement and general induration of the testis

Examination of skin lesions ordinarily included visualization and palpation. The skin lesions on the rabbits' back induced by intracutaneous inoculation have likewise been graded on a scale of 1 to 4 according to the *degree of induration. The grade 1 lesion is only slightly raised and infiltrated,* with ill-defined margins and often a scaly surface, while the grade 4 lesion is raised several millimeters above the normal level of the skin, with an induration approaching that of cartilage, and with rather sharply demarcated edges—in other words, the typical syphilitic chancre so well known in clinical medicine Grades 2 and 3 represent intermediate degrees of elevation and induration

The totals for each strain of initial testicular and skin lesions of all rabbits which survived long enough for lesions to develop in accordance with the foregoing classification are given in Table XLVI. Grades 1 and 2, which represent the least extensive lesions, have been grouped together for purposes of analysis, while grades 3 and 4, which represent advanced degrees of induration, are considered together for both testicular and intracutaneous lesions

Strains are grouped according to the particular syndrome from which they were originally isolated. It will be noted that for some strains, particularly those most recently isolated, the total number of animals observed has perhaps been too small to afford reliable statistical samples, but in most cases the numbers are adequate. Table XLVI includes columns for both testicular and skin lesions; these give the percentage represented by grades 3 and 4 lesions in the total for each strain On the basis of the data in these columns each strain is classified according to whether the percentage is in the upper, middle or lower third

Markedly indurated lesions have been associated for many years with strains of treponemes isolated from typical cases of venereally acquired syphilis occurring in the temperate zone; we have, therefore, designated

TABLE XLVI COMPARATIVE CHARACTERISTICS OF INITIAL TESTICULAR AND SKIN LESIONS OF RABBITS INOCULATED WITH VARIOUS STRAINS OF TREPONEMES

Group	Strain	Testicular lesions					Skin lesions			
		Number of animals	Number with grades of lesions		Percentage with grades 3 and 4	Number with periorchitis	Number of animals	Number with grades of lesions		Percentage with grades 3 and 4
			1 and 2	3 and 4				1 and 2	3 and 4	
Syphilis	Nichols	58	1	57	98	0	38	0	38 •	100
"	Chicago	109	0	109	100	1	30	2	28	93
"	Baghdad A	58	3	55	95	1	22	9	13	59
"	Baghdad B	51	11	40	78	7	20	5	15	75
"	Mexico A	17	15	2	12	6	7	3	4	57
Yaws	YD pre 1949	38	35	3	13	32	8	8	0	0
"	YD post 1949	61	7	54	89	7	3	0	3	100
"	Haiti A	42	38	4	10	18	13	11	2	15
"	Haiti B	62	34	28	45	21	19	13	6	32
"	Indonesia B	61	52	9	15	24	23	19	4	17
"	Samoa D	21	19	2	10	6	11	8	3	27
"	Samoa E	13	13	0	0	1	4	4	0	0
"	Samoa F	18	18	0	0	8	5	5	0	0
Bejel	Syria A	67	28	39	57	16	30	19	11	37
"	Syria B	61	18	43	70	11	21	11	10	48
"	Iraq B	70	22	48	69	11	37	28	9	24
Endemic syphilis	Bosnia A	53	33	20	38	4	22	12	10	45
"	Bosnia B	55	36	19	35	11	19	9	10	52
Dichuchwa	Bechuanaland C	11	6	5	45	2	4	2	2	50
"	Bechuanaland D	15	8	7	45	1	6	1	5	84

strains in which more than two-thirds of the inoculated animals show this type of lesion as type Sr (" S " for syphilis and " r " denoting that the characteristic is observed in rabbits). By the same token, strains in which at least two-thirds of inoculated rabbits show conspicuous absence of induration, a characteristic which has heretofore been associated with strains isolated from typical cases of yaws, have been designated the Yr type. Strains occupying an intermediate position in this respect have been designated the Mr type. It will be recognized, of course, that these categories have been more or less arbitrarily established. In view of the characteristic lesion noted in rabbits infected with cuniculi strains (see Chapter 2) a fourth, or C type, might be recognized

Results

Analysis of testicular lesions. When this grouping is applied to the data for testicular lesions, it will be noted that of the 5 strains isolated from patients who presented a clinical and epidemiological picture of typical venereally acquired syphilis the Nichols, Chicago, Baghdad A and Baghdad B strains are classified as Type Sr; while the Mexico strain is known as Type Yr.

By contrast, among 7 strains isolated from patients with typical yaws, 6 belong to the Yr type and one to the Mr type. The YD-post-1949 strain will be singled out for special comment later. Of the 3 bejel strains 2 belong to the Sr type and one to the Mr type; while the 2 strains of non-venereal syphilis and the 2 strains of dichuchwa can all be characterized as the Mr type.

Analysis of skin lesions. In general, the initial treponemal lesions in the skin have the same distribution, although there are exceptions based on relatively small numbers of animals The Mexico strain for example, is classed as an Mr type in respect of skin lesions, and as a Yr type in respect of testicular lesions. Perhaps the major discrepancy in the two indices is in the Iraq B strain, in which the testicular lesions tended to be of the Sr type, while the skin lesions were of the Yr type. This situation may be due in part to the fact that this strain was selected for the preparation of TPI and agglutinating antigen and consequently was subjected to rapid passage by the intratesticular route. Rapid passage, when it can be accomplished, usually leads to the induction of lesions of greater extent and induration. Similar changes are usually evident in skin lesions induced by rapidly passed material, although in the case of Iraq B strains this did not seem to be the case.

Data for granular periorchitis. The striking lesion known as periorchitis, which was first described by Pearce & Brown,[10] has long been regarded as fairly characteristic of yaws. It has been interesting, therefore, in a large

series of animals inoculated with many different strains to observe the relative incidence of this type of lesion The characteristics of the lesion itself have already been described in Chapter 2.

In some instances the lesions of granular periorchitis were actually visualized in the excised testes, while in others they were identified by palpation Since in instances in which the testis was subsequently visualized the findings usually confirmed those obtained by palpation the data derived from either type of examination are combined in Table XLVI.

A noteworthy fact which emerged from these studies is that this type of lesion was observed at least once in animals inoculated with every newly isolated strain There is, however, great variation in the frequency with which it occurs among the rabbits of the different strains; it is quite clear that in general it occurs very frequently among the yaws strains and infrequently among most of the syphilis strains Indeed, the wide experience with other syphilis strains (see Tables IA, IB and IC, Chapter 1, pages 21-23) afforded additional support for this statement, although an animal with granular periorchitis was also occasionally observed among rabbits inoculated with those strains

It is evident from the data presented in Table XLVI that the frequent occurrence of granular periorchitis can be positively correlated with the frequent occurrence of testicular and skin lesions of grades 1 and 2, or those which show minimal degrees of induration. To continue the scheme of classifying each strain with respect to the occurrence of a particular characteristic, we have considered those strains in which granular periorchitis occurred in 30% or more of the animals inoculated intratesticularly as belonging to the Yr type, those in which the incidence was between 15% and 29% as belonging to the Mr type, and those in which the incidence was less than 15% as belonging to the Sr type Again, it will be recognized that these divisions have been more or less arbitrarily made.

The results of this classification are shown in Table XLVIII. In general there is an inverse relationship between the occurrence of granular periorchitis on the one hand and indurated testicular and skin lesions on the other The syphilis strains as a group have a low incidence of granular periorchitis, while the yaws strains have a high incidence; bejel, endemic syphilis, and dichuchwa strains seem to occupy an intermediate position in respect of all these characteristics

The Disease Picture in Hamsters

The general characteristics of treponemal infections in hamsters have been presented in Chapter 2. It was noted that in general each treponemal strain produced a disease picture which could be placed in one of three categories on the basis of involvement of the skin and lymph nodes following intradermal inoculation in the groin.

One group of strains (Sh type) gave rise to few if any skin lesions at the site of inoculation, but there was involvement of the regional and other lymph nodes to the extent that large numbers of treponemes could be demonstrated by darkfield examination in most animals. Another group of strains (Mh type) gave rise to extensive skin lesions, but treponemes were about as numerous in the lymph nodes as in the case of the previous category. A third and rare pattern (Yh type) was characterized by extensive skin lesions, often with distant metastasis, with few if any treponemes demonstrable in the regional or other lymph nodes.

The biological basis for these differences is obscure, but one or another of these patterns was quite consistently observed for each strain With those observations as a basis, the behavior of various strains of treponemes will be analysed from a comparative standpoint

The data to be presented are drawn from the observation of 533 hamsters inoculated with one or another of the newly isolated strains listed in Chapter 1 (Table Ic). The study was carried out in 1953, 1954 and the first half of 1955, during this period the method of inoculation, frequency of transfer, and manner of observation remained essentially the same

Pertinent data for 22 treponeme strains are summarized in Table XLVII: 18 strains were newly isolated ones, while 4 strains had been isolated from their natural host some years previously. Two strains did not clearly fit into any one of these groups and have been left unclassified

Six strains—Nichols, Chicago, Mexico A, Baghdad A, Baghdad B, and YD-post-1949—gave the Sh type of reaction. With the exception of the YD strain all of them will be recognized as the strains which were originally isolated from typical cases of venereally acquired syphilis

It should be mentioned in passing that while a small proportion of the hamsters infected with these strains showed local lesions, these lesions were almost universally quite small and of short duration In other words there was a qualitative as well as a quantitative difference between the strains in Group Sh and those in the other two groups There was a total absence of extending skin lesions. The local lymph nodes, however, showed just as many treponemes and in just as high a proportion of animals as did the lymph nodes of the animals included in Group Mh.

Ten strains are included in Group Mh. Haiti A, Haiti B, Indonesia B, Syria B, Iraq B, Bosnia A, Bosnia B, Bechuanaland C, Bechuanaland D, and YD-pre-1949. Referring back to the description of the source of these strains (see Chapter 1, Table Ic), it will be noted that four of them were obtained from typical cases of yaws, two from Haiti, one from Jamaica and one from Indonesia. Included in addition, however, are two strains isolated from patients with bejel. Syria B and Iraq B; two strains isolated from patients with endemic syphilis: Bosnia A and B; and two strains isolated from cases of so-called dichuchwa: Bechuanaland C and D.

TABLE XLVII COMPARATIVE CHARACTERISTICS OF TREPONEME STRAINS IN HAMSTERS

Group	Strain	Number of animals observed	Number of animals with			Classification type [a]
			Local lesions	Extended lesions	Lymph node involvement	
Syphilis	Nichols	24	4	0	18/20 [b]	Sh
"	Chicago	46	6	0	41/41	Sh
"	Baghdad A	26	8	0	20/25	Sh
"	Baghdad B	16	2	0	14/16	Sh
"	Mexico A	21	4	0	21/21	Sh
Yaws	YD pre 1949	24	24	5	—	Mh [c]
"	YD post 1949	21	1	0	16/21	Sh
"	Haiti A	22	14	6	16/21	Mh
"	*Haiti B*	*52*	*51*	*10*	*23/24*	*Mh*
"	Indonesia B	26	25	23	17/20	Mh
"	Samoa D	43	41	25	3/34	Yh
"	Samoa E	42	42	26 [d]	3/26	Yh
"	Samoa F	44	43	28 [d]	0/28	Yh
Bejel	Syria A	16	10	8	7/7	Mh
"	Syria B	20	14	6	15/19	Mh
"	Iraq B	8	6	1	7/7	Mh
Endemic syphilis	Bosnia A	22	21	12	11/14	Mh
"	Bosnia B	22	14	9	16/18	Mh
Dichuchwa	Bechuanaland C	20	12	5	15/18	Mh
"	Bechuanaland D	19	19	12	15/17	Mh
Pinta	Undesignated	36	0	0	3/36	Unclassified
Cuniculi	Cuniculi A	25	1	0	11/23	Unclassified

a

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TABLE XLVIII SUMMARY OF STRAINS ACCORDING TO CHARACTERISTICS OF INITIAL TESTICULAR AND SKIN LESIONS IN RABBITS, AND TYPE OF DISEASE IN HAMSTERS

Human disease	Strain	Category of rabbit reaction			Disease in hamsters group [c]
		Testicular [a]	Skin [a]	Periorchitis [b]	
Syphilis	Nichols	Sr	Sr	Sr	Sh
”	Chicago	Sr	Sr	Sr	Sh
”	Baghdad A	Sr	Mr	Sr	Sh
”	Baghdad B	Sr	Sr	Sr	Sh
”	Mexico	Yr	Mr	Yr	Sh
Yaws	YD pre 1949	Yr	Yr	Yr	Mh [d]
”	YD post 1949	Sr	Sr	Sr	Sh
”	Haiti A	Yr	Yr	Yr	Mh
”	Haiti B	Mr	Yr	Yr	Mh
”	Indonesia B	Yr	Yr	Yr	Mh
”	Samoa D	Yr	Yr	Mr	Yh
”	Samoa E	Yr	Yr	Yr	Yh
”	Samoa F	Yr	Yr	Yr	Yh
Bejel	Syria A	Mr	Mr	Mr	Mh
”	Syria B	Sr	Mr	Mr	Mh
”	Iraq B	Sr	Yr	Mr	Mh
Endemic syphilis	Bosnia A	Mr	Mr	Sr	Mh
” ”	Bosnia B	Yr	Mr	Mr	Mh
Dichuchwa	Bechuanaland C	Mr	Mr	Mr	Mh
	Bechuanaland D	Mr	Sr	Sr	Mh

[a] Type S — more than $^2/_3$ indurated lesions
Type M — intermediate
Type Y — less than $^1/_3$ indurated lesions
[b] Type S — incidence of periorchitis under 15%
Type M — incidence of periorchitis 15%-29%
Type Y — incidence of periorchitis 30% or higher
[c] Type S — Local lesions rare, lymph nodes commonly positive
Type M — local lesions common, extension of lesions frequent, lymph nodes commonly positive
Type Y — local lesions common, extensions very frequent, lymph nodes rarely positive
[d] Classification questionable because lymph nodes were not examined for treponemes

Comparative Histology of Strains of Treponemes in Rabbits

The histologic features of lesions produced by infection with the Nichols strain of syphilis were presented in Chapter 2 Ferris & Turner [4] in 1938 described the histologic appearance of cutaneous lesions of yaws and syphilis in rabbits, these observations being based on a study of some 270 lesions

In the hamsters included in this group, skin lesions occurring at the site of inoculation were not only more frequent, but with few exceptions were larger and much more persistent than in the animals in Group Sh There was also a noteworthy tendency for these lesions to extend peripherally and eventually to involve wide areas of skin. In all of these lesions active treponemes could be demonstrated readily during the entire period in which the lesions were present, treponemes could likewise be easily demonstrated in the inguinal lymph nodes of most of the animals.

Group Yh comprises three strains, all of which were isolated in Western Samoa The disease picture induced in hamsters differs from that described above, in that associated with a high frequency of persistent local skin lesions with an unusual tendency to extend is a surprising infrequency of lymph-node involvement The numbers of animals affected are much too large for this to be a variation due to chance alone. Again we can offer no suggestion as to the probable biological basis for this phenomenon

Two strains of treponemes have been placed in the unclassified category because they did not appear to belong clearly to any one of the other groups, and existing data were too meager to warrant the establishment of one or more additional groups. The cuniculi A strain induced a local skin lesion in only one of 25 hamsters inoculated, treponemes were demonstrated in the regional lymph nodes in about half of the inoculated animals, and then only in relatively small numbers.

Our experience with the Mexico strains of pinta treponemes has already been recorded in Chapter 2. Suffice it to say that infection could be proved in only 3 of 36 hamsters inoculated, and then only by the demonstration of a relatively small number of treponemes in the regional lymph nodes

Comparison of Behavior of Treponemal Strains in Hamsters and Rabbits

[illegible] characteristics of the trepo-

both in hamsters and in rabbits. The Mexico A strain is an exception, since, although in hamsters it behaved in the same way as other syphilis strains, in rabbits it conformed somewhat to the yaws pattern.

The situation in respect of the other non-venereal treponematosis strains is interesting in that all of them conformed to the pattern usually found with yaws in hamsters, but in rabbits they induced a type of reaction which, viewing the animals as a group, was intermediate between reactions produced by the syphilis strains on the one hand and the yaws strains on the other.

While we have little understanding of the basis for these differing patterns, the subject will be discussed further in Chapter 10.

It was noted that polymorphonuclear leukocytes were present only in considerable numbers in lesions which had undergone ulceration, and hence were relatively inconspicuous in the lesions of yaws. The degrees of inflammatory reaction observed are graphically illustrated in Fig. 13

In the silver-stained preparations treponemes were found abundantly in many of the syphilis lesions, but rarely in the yaws lesions, and then chiefly in lesions taken during the early stages of the infections. These features are also graphically represented in Fig 13 From this study[4] it was concluded that the histologic changes were qualitatively the same in the lesions of yaws and syphilis.

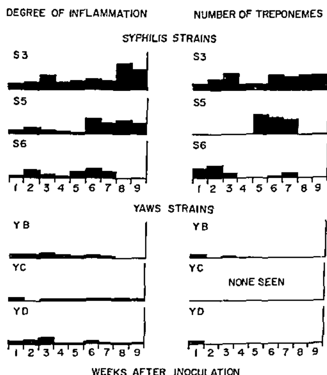
Matsumoto[8] and his associates have likewise compared the histological appearance induced by strains of syphilis with that produced by two strains of yaws from Palau and one from Saipan, and have noted particularly the distribution of the " myxomatous " changes The skin lesions in yaws were described as differing from those of syphilis " only in degree " The testicular lesions and generalized lesions also were not distinguishable from syphilis although the most characteristic lesions of yaws were in the testes. These, however, were lesions of granular periorchitis, recognized by their macroscopic appearance rather than by any specific histologic difference Myxomatous changes were also identified in many of the yaws lesions although they were less extensive than in the syphilis lesions " In brief the histologic features of the localized and generalized lesions of frambesia in rabbits present striking resemblances to those of syphilis in the same animals "[8]

The recently isolated strains of syphilis, endemic syphilis, yaws and bejel studied in this laboratory have offered an opportunity to re-evaluate the histopathologic features of experimental treponemal infection in the light of some of the ideas of treponemal infection presented in this monograph. For each of the strains isolated, sections of skin lesions and sections of testes were routinely prepared by fixing in neutral solution of formaldehyde (4%), embedding in paraffin, and staining with hematoxylin and eosin Many lesions from rabbits with their respective infections in various stages of evolution were examined, particularly infections with those strains longest in the laboratory, including syphilis strains Baghdad A, Baghdad B, and Chicago, endemic syphilis strains Bosnia A and Bosnia B; bejel strains bejel A, bejel B and Iraq B, and yaws strains Haiti A, Haiti B and Indonesia B The following remarks will apply chiefly to these strains

Since the material was often obtained when the animals were sacrificed, or when transfers were to be made, the majority of the lesions were from well-developed infections, and because of variation in the size of the inoculum and other uncontrolled factors, the sections are not strictly comparable even when lesions of the same age are compared Nevertheless the total material probably gives a representative picture of the various experimental infections.

from yaws strains YB, YC, YD and syphilis strains S3, S5, and S6. Specimens were resected after 1, 2, 3, 4 and 6 weeks, stained by hematoxylin and eosin, and with silver impregnation by the Warthin-Starry technique Particular attention was paid to the abundance of cellular elements, and to the number of treponemes present at various stages of the infection for each strain.

FIG 13 DEGREE OF INFLAMMATION AND NUMBER OF TREPONEMES IN LESIONS OF SYPHILIS AND YAWS



Reproduced from Ferris & Turner,[4] *by kind permission of the editors of* Archives of Pathology

With each of the syphilis strains in the early stages diffuse infiltration of mononuclear cells, chiefly lymphocytes, was seen, and with the yaws strains a similar but considerably lesser reaction was noted After 3 weeks the yaws lesions became inconspicuous microscopically as well as macroscopically, while most of the syphilis lesions developed various degrees of necrosis,

It was noted that polymorphonuclear leukocytes were present only in considerable numbers in lesions which had undergone ulceration, and hence were relatively inconspicuous in the lesions of yaws. The degrees of inflammatory reaction observed are graphically illustrated in Fig. 13

In the silver-stained preparations treponemes were found abundantly in many of the syphilis lesions, but rarely in the yaws lesions, and then chiefly in lesions taken during the early stages of the infections These features are also graphically represented in Fig. 13 From this study [4] it was concluded that the histologic changes were qualitatively the same in the l ... and syphilis.

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The recently isolated strains of syphilis, endemic syphilis, yaws and bejel studied in this laboratory have offered an opportunity to re-evaluate the histopathologic features of experimental treponemal infection in the light of ... nemal infection presented in this monograph ... and sections of testes ... ormaldehyde (4%), embedding in paraffin, ... in and ... Many lesions from rabbits with their respective infections ... various ... and particularly infections with those strains ... and A Baghdad B, ... strains ... sia

B The following ...

Since the material was often obtained when ... ed, or when transfers were to be made, the majority of the lesions were from well-developed infections, and because of variation in the size of the inoculum and other uncontrolled factors, the sections are not strictly comparable even when lesions of the same age are compared Nevertheless the total material probably gives a representative picture of the various experimental infections.

From this material it can be stated that none of the strains shows any qualitative difference in the character of the histologic reaction No strain or species can be identified solely on the basis of its microscopic appearance. The three portions of the histologic reaction described for the Nichols strain (see Chapter 2) may be present for each strain, namely, mucoid material, mononuclear reaction, and necrosis, although in quantitatively different ratios

Areas containing mucoid material were most evident with the syphilis strains, particularly Chicago but also Baghdad A and Baghdad B Mucoid material was frequently encountered in animals infected with Bosnia A, Bosnia B, bejel A, bejel B, and Haiti B treponemes. It was more difficult to find but nevertheless noted in an occasional section from Iraq B and Haiti A animals For each strain the occurrence and approximate amount of mucoid material could be correlated with the clinical prominence of the lesions in that strain. The distribution of the mucoid material was not characteristic for any strain. Sometimes it surrounded tubules of the testes or blood vessels, at other times it was infiltrating areas such as the interstitial tissue of the testis, or the connective tissue of the skin Since identification was made on the basis of the disruption of other structures, small amounts of this material may have been overlooked.

The mononuclear cell infiltration is the predominant feature of most lesions. Infections with strains in which mucoid substance is most prominent in the early stages usually exhibit the most extensive lymphocytic reaction in later stages.

The polymorphonuclear reaction goes with ulceration and is therefore found most often in animals infected with those strains which produce the largest lesions In general, the lesions of grades 3 and 4, more commonly found in animals inoculated with strains of syphilis, are those associated with macroscopic ulceration, histologic evidence of necrosis and polymorphonuclear infiltration.

The basis for the differing histological picture in respect of the presence of mucoid material is not known for certainty. A reasonable assumption is that different strains of treponemes produce different amounts of mucoid material On the other hand, the accumulation of this material may merely reflect the presence of large numbers of treponemes It is also conceivable that the mucoid material produced by various strains is broken down at different rates within the rabbit host

[illegible] newly isolated strains [illegible]

in the rabbit do not [illegible] and only those infections which exhibit necrosis clinically show the response of acute inflammation microscopically. As a result the predominant reaction

of the characteristic indolent infection of many strains is often primarily a mononuclear infiltration Whether the decreased amount of mucoid material represents less hyaluronic acid per individual treponeme, or faster destruction of the material, is not known We are inclined to the view, on the basis of collateral evidence, that different strains of treponemes do vary in their capacity to produce hyaluronic acid, and that this characteristic may be the fundamental basis for the differences between strains.

Variation and Mutation of Treponemal Strains

Involved in the entire problem of the comparative characteristics of strains of treponemes is the question as to whether the observed characters are fixed, or whether each strain has an inherent capacity for variation. In nature, the environment of a given geographical area remains fairly constant, and it is understandable that local treponemal species have become stabilized during passages in their natural hosts over many decades

In the laboratory, while an essentially stable environment can be established, it may differ in many important respects from that natural environment in which a particular strain has hitherto been propagated. Thus, on theoretical grounds at least, the stage may inadvertently be set for the occurrence of variation and even mutation, a phenomenon which in the light of accumulating knowledge appears to be an inherent characteristic of living things.

We have only three sorts of observations bearing on this general question One has to do with the retention of pathogenicity for man of treponemal strains after passage in animals for many years A second stems from the observation that with repeated animal passage under conditions that favor the parasite, such as rapid transfer in rabbits and cool environmental temperature, yaws strains tend to assume many of the characteristics of syphilis strains Thirdly, direct observations in the laboratory of certain yaws strains strongly suggest that true mutations have occurred

Persistence of Pathogenicity of Laboratory Strains for Man

The fact that strains of treponemes originally derived from humans may remain pathogenic for humans after long periods in laboratory animals has been well established by a series of accidental laboratory infections These have been reviewed by Wakerlin,[12] and more recently by Durel & Sausse.[3]

Infections following accidental human inoculation with laboratory strains were reported by Metchnikoff and Roux in 1906, and by Buschke in 1913, each from an infected monkey, and by Graetz and Delbanco in 1914, Levaditi and Marie in 1919, and Grayhyll in 1924 from rabbit material (cited by Durel & Sausse [3]) Levaditi & Vaisman,[6] however, partly on the

basis of the inoculation of monkeys with rabbit material believed that the Truffi strain had lost most of its virulence in the course of its long passage in rabbits Wakerlin[12] in 1932 reported a human infection with the Nichols strain of *T pallidum*. This strain was isolated by Nichols & Hough[9] in 1912 and at the time of the human infection had been passed continuously in rabbits for 13 years.

In 1933 and 1943 two additional instances of accidental laboratory infection with the Nichols strain were observed in Baltimore. The details of these infections were not published, but in each instance the source of the infection seems to have been clearly established

More recently, two cases have been reported by Durel & Sausse,[3] the disease occurring as a result of infection with the Nichols strain 42 years after the original isolation in 1912, during which time the strain had been continuously passed in rabbits; in these instances the source of the infection was a sub-strain, obtained from this laboratory, with which virtually all the experiments reported in this monograph were performed.

Variation in Strains of Treponemes

It is a common observation that many species of bacteria and viruses as well as the pathogenic treponemes, become better adapted to laboratory animals with continued passage This perhaps may be regarded as a form of variation, in the ordinary genetic sense. Indeed, the studies of Burnet[1] on strains of influenza viruses, in which it was possible to identify certain genetic markers, suggest that these adaptive mechanisms involve true mutational phenomena

Much of the information on the behavior of strains of treponemes in laboratory animals has come from general impressions formed by investigators over a period of years, rather than from more precise data; nevertheless the value of such impressions should not be wholly discounted. With ordinary passage of strains of treponemes one is impressed perhaps by the stability of characteristics rather than by changing patterns. For example, the senior author when working in Chesney's laboratory had the opportunity to observe the behavior of five strains of syphilis treponemes during many passages in rabbits Two of these strains, the Truffi strain isolated originally in Europe, and strain F, isolated by Chesney, Kemp & Resnik[2] regularly induced an extensive disease picture in rabbits, characterized by extremely large indurated initial lesions, and generalized lesions in a high proportion of rabbits. At the other end of the scale were two strains, designated C[2] and H,[5] which characteristically induced a comparatively slight initial lesion, generalized lesions were less frequent. The Nichols strain induced a disease picture intermediate between these two extremes (Chesney & Turner, unpublished observations, 1931) It can be said, however, that none of these infections resembled the yaws pattern outlined previously.

Likewise, in the case of the newly isolated strains studied in this laboratory over the past few years, most of the strains after one or two passages in rabbits tended to behave in much the same fashion in passage after passage, unless subjected to special manipulation The Chicago syphilis strain, for example, from the first passage was highly virulent for rabbits, inducing almost without exception large indurated mucoid initial lesions At the other end of the scale, the Indonesia B strain and the Samoa yaws strains have from the beginning been difficult to propagate in rabbits and the character of the lesions induced has not appreciably changed with repeated rabbit passage.

On the other hand, certain strains have been singled out for rapid passage with a view to obtaining satisfactory antigens for use in the immobilization *or the agglutination test The Iraq B strain of bejel treponemes, the Haiti B* strain of yaws and the cuniculi A strain have been handled in this manner. In each instance it was possible, particularly with concomitant administration of cortisone, to induce larger testicular lesions with shorter incubation periods and yielding more treponemes than before While there was no evidence that any permanent alteration in the essential characteristics of these strains had occurred, in general the type of lesion shifted toward the syphilis end of the spectrum.

In general, when a change in the character of a strain has been observed, this has been an alteration of a yaws strain toward the syphilis type. It should be noted that the yaws strains were usually being propagated either in a naturally cool environment or in one artificially cooled, as in our own *laboratory It is possible that the temperature influenced these changes* We have, therefore, attempted to induce changes in the opposite direction in two highly virulent syphilis strains—Nichols and Chicago—by propagating them in rabbits subjected to continuously high environmental temperatures (29-31°C) Simultaneously, these same strains are being carried in rabbits subjected to cool temperatures (18-21°C) Transfers from each group are made usually on the same day This experiment has now been in progress for nearly two years. As anticipated from the results of experiences reported in Chapter 3, the lesions in the warm-room animals have been smaller and of shorter duration and the strains have been much more difficult to maintain. Indeed, the warm-room line of the Chicago strain was lost in the 14th passage and it has been necessary to resort to the use of 10th-passage frozen material to perpetuate this sub-line

After the experiment had been in progress for about one year, the 10th passage of each strain in both the warm-room and cold-room animals was inoculated intracutaneously, in doses of 500 treponemes at each site, into groups of normal rabbits, all of which were maintained in the cold-room. This experiment, in brief, revealed that the incubation period and the size and character of the lesions produced by sub-lines of treponemes, which had been propagated at high environmental temperatures for a year. did

not differ from the incubation period and lesions produced by the sub-lines propagated in the cold. Obviously, little or no change had occurred during the first year[a]

Mutation-Like Changes in Yaws Strains

It is difficult to know at what point an observed change may be regarded as a true mutation as opposed to a variation, using the terms in their accepted genetic sense The changes cited above have been relatively slight and probably largely reversible Other observations, cited below, indicate, however, that changes may occasionally occur which in magnitude and persistence resemble genuine mutations

Manteufel & Herzberg[7] a number of years ago observed that a gradual shift had taken place in the character of the lesions produced in rabbits by a yaws strain being propagated in their laboratory, the lesions which originally were typical of yaws eventually came to resemble those induced by their syphilis strain.

Two other yaws strains observed in Baltimore—one propagated in Chesney's laboratory and the other in the authors' laboratory—likewise apparently underwent permanent alteration during passage in rabbits.

Strain Y9 (Chesney)

The following account of the behavior of the Y9 strain of yaws is quoted from an unpublished manuscript written by Dr Alan M Chesney, shortly after the observations were made, and reviewed by him in collaboration with the authors of this monograph during 1955

"In the winter of 1929-30 Dr T B Turner inoculated several series of rabbits in Haiti with material obtained from lesions of patients with yaws He also inoculated a series of rabbits at the same time with material from a patient with syphilis In this way eight strains of yaws treponemes and one strain of syphilis treponemes were successfully isolated from patients in Haiti and were brought to the United States for study.

"Of the eight yaws strains isolated in Haiti, five were subsequently discarded for reasons of economy, and two of the remaining three were lost in the 6th animal passage The surviving strain was known as "Y9" The syphilis strain obtained in Haiti was called "Strain K".

"In order to propagate the strains, transfers were made from rabbit to rabbit by intratesticular inoculation at frequent intervals.

[a] Similar tests made on the 20th passage of each strain after the end of the second year yielded a similar result

"The essential feature of this experimental yaws infection produced by intratesticular inoculation of rabbits was the evolution of localized miliary granulomatous lesions in the body of the testis, the tunic or the epididymis, and the absence of diffuse orchitis, of scrotal edema, and of metastatic lesions involving the skin, bones or eyes In an occasional animal a few firm nodules as large as 3 mm in diameter developed in the body of the testis, or a solitary nodule appeared in the tunic of the subscrotal tissue, but these were exceptional instances during the period covered by the second paper of this series, [11] that is, January 1930 to June 1932

"It was not always easy to be certain when the animals inoculated with the yaws organisms had come down with the infection, especially if they were inoculated in the summer months, so that it became the practice to carry a fairly large number of yaws-inoculated rabbits in the laboratory long after they had ceased to show active manifestations of the disease, in order to guard against losing the strain

"In the late autumn of 1932, nearly three years after the original isolation of the yaws strain, it looked as if that strain (Y9) had been lost The rabbits which had been inocu-

[illegible]

but were not showing any active yaws lesions at the time Accordingly, during the months of December 1932 and January 1933 transfers of testicular material were made from four yaws-latent rabbits and lymph-node transfers from two of these same animals. In addition, lymph-node transfers were made from three other yaws-latent animals As a result of these transfers *five* different substrains of our yaws strain (Y9) were recovered All of these substrains were in the ninth animal passage when recovered For one or another reason all except one of these substrains (Y9-2) were discarded

[illegible]

Y9C

[illegible]

of *T pallidum* isolated in the temperate zone

"Although no account was taken of the incidence or character of the metastatic lesions produced by these strains when it came to the question of classifying the disease produced by them, nevertheless it is of interest to examine the incidence of such lesions in the animals studied. Reference to Table [XLIXA] shows that generalized lesions were comparatively rare in animals inoculated with the parent yaws strain (Y9), being found in only 7, or 11 3 per cent, of all the animals in which disease manifestations occurred Moreover the only type of generalized lesion which was produced by this strain was that of orchitis involving the opposite testis, for no lesions involving the skin, the bones or the eyes were observed. This state of affairs is in marked contrast with that which obtained in the case of the Haitian syphilis strain (K) which showed a high incidence of metastatic lesions, actually 108 in 146 animals, or 73.9 per cent. Moreover in the case of this strain there was not only metastatic orchitis but lesions involving the skin, bones and eyes In other words, from the standpoint of production of metastatic lesions in the rabbit the Haitian syphilis strain behaved exactly like any strain of *T pallidum* isolated from patients with syphilis in the temperate zone.

TABLE XLIXA SUMMARY OF THE TRANSITION OF THE CHARACTERISTICS OF STRAIN Y9 (CHESNEY) DURING INTRATESTICULAR PASSAGE IN RABBITS *

Characteristics	Yaws strain			Syphilis strain
	Y9 (Chesney) Parent strain (62 animals)[a]	Y9B Sub-strain (133 animals)[a]	Y9C Sub-strain (127 animals)[a]	K (Chesney) (146 animals)[a]
Yaws like lesions	54	55	68	0
Syphilis like lesions	5	53	32	142
Lesions equivocal	3	25	27	4
Orchitis in uninoculated testis	7	64	73	100

* Adapted from Chesney, unpublished observations, 1955
[a] Numbers denote animals showing indicated type of lesion

"The yaws substrains occupied a position midway between the parent yaws strain and the Haitian syphilis strain in respect of the occurrence of metastatic lesions Thus, the [illegible]

of these substrains, but no lesions involving the eyes were observed."

Strain YD (Turner and Hollander)

In this laboratory a trend from the yaws type of reaction toward the syphilis type has been noted with several strains during continued passage but in no case so strikingly as with our strain YD originally isolated in Jamaica in 1932 (see Chapter 1, Table IA, page 21)

TABLE XLIXB SUMMARY OF THE TRANSITION OF THE CHARACTERISTICS OF STRAIN YD DURING PASSAGE IN RABBITS

		YD pre 1949			YD post 1949
		Passages 1 15 (1936 46)[a]	Passages 16 25 (1946-49)[a]	Passages 26 36 (1949 50)[a]	Passages 27-42 (1952 53)[a]
Rabbit reaction	Yaws like granular periorchitis	14	11	1	0
	Indurated nodular syphilis like	0	10	26	28
Hamster reaction	Number of transfers from rabbits	0	0	3	6
	Number of animals			17	30
	Number of lesions			24	2
	Number of extensions			7	0
Classification		Yr	Mr	Sr Yh	Sr Sh

[a] Numbers denote individual animals Some animals are not included because of insufficient data

The passages of YD can be divided into three periods on the basis of the strain's behavior in rabbits and hamsters (Table XLIXB) During the first 15 generations every animal exhibited granular periorchitis and only slightly indurated testicular lesions (the Yr type), during the next 10 generations the reaction was not uniform· some animals showed a granular periorchitis, while others developed indurated lesions resembling syphilis (the Mr type), after the 25th generation, the reaction was uniformly one of induration and nodule formation without granular periorchitis, and indistinguishable from syphilis in the rabbit (the Sr type)

It will be instructive to follow in some detail the changes observed after the 25th passage made on 7 December 1949, since two sub-lines of strain YD can be identified As noted above, a number of rabbits inoculated prior to this date had shown markedly indurated testes, although granular periorchitis was frequently observed too On 7 December 1949 transfers were made to both rabbits and hamsters from the right testis of rabbit No 26-81, both testes of this animal were enlarged and indurated but with granular surfaces

The 3 rabbits inoculated with material from the right testis of No 26-81 developed lesions characteristic of syphilis, while a group of 10 hamsters inoculated with this same material exhibited a reaction of the yaws type (Mh) in that 9 of the 10 had lesions at the site of inoculation, and in 4 the

lesions extended beyond the local area Three subsequent serial passages in hamsters showed the same type of reaction

On 9 December 1949, transfers were made from the left testis of rabbit No. 26-81 to 4 rabbits, each of which also developed syphilis-like lesions Ten rapid serial passages were then made in rabbits, beginning with one of these animals. Without exception the testicular lesions in these successive passages resembled the syphilis type After the 10 passages of this sub-line, 2 more hamsters were inoculated and again these developed lesions characteristic of the yaws or Mh type of reaction.

A second sub-line originated in the following manner. Three of the animals inoculated from the left testis of No. 26-81—the same material that inaugurated the first sub-line—were kept for serial bleedings over a period of months At the end of 27 months only one animal survived; lymph-node transfers were made from this animal to determine the presence or absence of infection The single rabbit inoculated with these nodes developed a nodular type of testicular lesion and a granular periorchitis Transfers from this animal were made to rabbits and were propagated in 15 rapid serial passages All of these animals developed syphilis-like lesions without the occurrence of granular periorchitis Of particular interest is the fact that hamsters inoculated from various passages of this series all showed a reaction of the Sh or syphilis type, of 30 hamsters inoculated, only 2 developed transient questionable local lesions, while 28 hamsters showed no lesions, of 24 of the latter examined, 21 showed treponemes in the lymph nodes. These same characteristics have persisted in both rabbit and hamster passages until the present time

Discussion on Variation in Strains of Treponemes

Judged by the rather inadequate criteria by which we classify strains as yaws-like or syphilis-like, these two strains, Y9 (Chesney) and YD (Turner and Hollander), may be said to have undergone apparently irreversible changes from the yaws to the syphilis type This appeared to have been a step-wise process, including stages during which the treponemes exhibited the features of both species. In each instance the final change occurred during a long-standing infection in a single rabbit, probably in its lymph nodes. It is a familiar fact that yaws treponemes are present less frequently in rabbits' lymph nodes than are syphilis treponemes, it might be postulated that this environment is particularly favorable to the survival of those treponemes having properties that are biologically closest to the syphilis type

It should be noted that, in theory, if mutations occur a strain of treponemes could become *genetically heterogeneous*, and the heterogeneity could be perpetuated in serial passages, which are ordinarily made with large populations of treponemes. On the other hand, a situation where

syphilis treponemes can differentially survive might allow for the segregation of treponemes with syphilis characteristics from populations which are predominantly of a different character Whether or not a process of this sort is taking place in nature is not known.

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Chapter 8

ANTIGENIC RELATIONSHIP BETWEEN STRAINS OF TREPONEMES

It is commonplace in microbiology that strains of a particular organism which clearly resemble one another in many important features may often nevertheless exhibit readily detectable antigenic differences. The extent of the difference, as well as its biological significance, varies from organism to organism. In part the identification of differences in antigenic structure is dependent on the precision of the methods available for detecting variations of this sort

In the case of the *Salmonella* group of organisms, well over 100 species have been identified on the basis of differences in one of several antigenic components of the organism. The pneumococci and streptococci have been separated into many different types on the basis of variation in antigenic pattern. Viruses, too, such as those of poliomyelitis, influenza and the adenoidal-pharyngeal-conjunctival (APC) group have been separated into immunological types by means of neutralization tests

On the other hand some groups of bacteria and viruses seem to be homogenous from an antigenic standpoint For example, while different strains of *Mycobacterium tuberculosis* vary in virulence, no parallel differences have been detected in their antigenic structure, nor have immunological differences been found among different strains of yellow fever or rabies virus This, of course, may merely reflect inadequacies of the experimental methods.

With reference to the *Treponema*, few data of a conclusive nature are available concerning their immunological relationships Since this is a problem in which our laboratory has been interested for a number of years, the accumulated data bearing on this question will be presented in some detail.

Methods Available for Studying Immunologic Relationships

In these studies the investigator is handicapped in applying recognized immunochemical procedures, because pathogenic treponemes can be grown only *in vivo*. Since the amount of host tissue material greatly exceeds

the treponemal mass, it is difficult to obtain relatively clean antigenic components. Moreover, the usual antibody absorption methods are not practicable, because of the difficulty of producing the large quantities of organisms required for absorption.

Certain procedures are available, however, principal among which are the following. (*a*) cross-immunity experiments—in which rabbits are infected with one strain of treponeme, the infection is permitted to evolve until immunity to that strain develops, and the animals are then challenged with *another strain. Failure to develop lesions upon the second inoculation*, or modification of the second infection under properly controlled conditions, signifies some antigenic similarity between the strains, and (*b*) antigen-antibody studies—in which serum antibody levels are compared, by utilization of the treponemal immobilization test or the treponemal agglutination test.

Evidence Obtained from Cross-Immunity Studies

Considerable data are available from other laboratories to show that rabbits infected with one strain of *Treponema* obviously develop a higher degree of immunity to reinfection with that strain than to reinfection with other strains. The results of these earlier studies are entirely valid, but usually the test procedure favored the challenging organism, and tended to accentuate differences rather than to reveal similarities For example, in most instances both first and second inoculation were made into the rabbits' testes, and very large challenge doses were employed Moreover, for the most part, those studies were made with relatively few strains, since the prime objective was something other than a study of the immunological relationship of specific strains of treponemes

In our studies we have throughout induced the initial infection by the intratesticular route, and after an interval of 3-4 months, have challenged these animals by intracutaneous inoculation The challenge inoculation was commonly made with a measured number of treponemes, and always intradermally on the clipped back of the animal Invariably the challenge inoculation was made into more than one site, usually into 4, so that in the event that specific lesions appeared the pattern would be unmistakable This proved to be helpful in those instances when slight or evanescent lesions developed from challenge inoculation.

An interval of 3-4 months after initial infection was chosen because the studies referred to previously indicated that resistance to challenge inoculation with the same strain reaches its maximum by 3 months Since it was desirable to keep such cross-immunity tests as simple as possible, the test animals were not subjected to treatment prior to challenge inoculation.

Development of lesions following intracutaneous inoculation of the challenge strain has been selected as an index of immunity, in the recognition

that this is indicative of a certain level of immunity only, since, as was observed in Chapter 5, even when the same strain is used for challenge refractoriness to reinfection develops in only about one-third of the animals. Nevertheless, failure of test animals to develop lesions at the site of challenge inoculation, when controls regularly develop definite treponemal lesions, is certainly indicative of a substantial degree of cross-immunity and is to be interpreted as reflecting some measure of antigenic relationship between the two strains.

Throughout our studies the Nichols strain of *T. pallidum* has been used as the reference strain, and two approaches have been employed Animals were either infected with the Nichols strain and challenged with various other strains, or they were initially infected with one or another strain and challenged with the Nichols strain. As a rule four animals were inoculated as a group, although deaths from intercurrent infections sometimes reduced this number to three or even rarely to two. In such instances the tests were usually repeated.

In the early experiments, rabbits were first challenged with the homologous strain to demonstrate that each was in fact immune. Since only rarely did an animal exhibit lack of immunity after three months to the homologous strain, this procedure was discontinued on the basis of the finding that the information thus obtained was not commensurate with the extra time, labor, and cost involved. We feel that it is justifiable to assume that all so-called immune animals are actually immune to the homologous strain, bearing in mind the possibility that strains may be discovered at some future time which behave otherwise.

Cross-immunity tests on a group of syphilis strains

The strains included in the first series of tests were the ones which had been isolated either in Baltimore or in Jamaica. (See Chapter 1.)

In the first experiment, a series of rabbits was inoculated intratesticularly with one or another newly isolated strain, and after 3-4 months challenged with the Nichols strain Challenge inoculations were made intracutaneously at each of 4 sites with material containing 500 treponemes

The results are shown in Table L It will be noted that all strains, except L.W.38, F.R.47 and St Louis 47, induced in rabbits a high degree of refractoriness to challenge with the Nichols strain.

Cross-immunity tests were made on some of these same strains, as well as on a number of others by the alternate method, in which rabbits were first infected with the Nichols strain, and 3-4 months later challenged with one of the other strains In this series of tests 500 treponemes were inoculated intracutaneously at each of 4 sites The results are shown in Table LI.

It will be noted that the Nichols strain induced a high degree of refractoriness to most of the strains in this series. Again, with strains L.W.38

TABLE L. RESULTS OF CHALLENGE INOCULATION OF 500 "T PALLIDUM" OF THE NICHOLS STRAIN IN RABBITS PREVIOUSLY INFECTED WITH VARIOUS STRAINS OF SYPHILIS TREPONEMES

Immunizing strain	Result of challenge inoculation [a]	Immunizing strain	Result of challenge inoculation [a]
None	22/22	L W 39	0/4
Nichols	0/15	M J 40	0/4
S6	0/4	L J W 47	1/6
S10	0/3	A H 47	0/5
L W 33	5/5	F P 47	2/3
M S I 39	2/7	St Louis 47	5/5
M S II 39	0/4	H W 48	1/5
M S S 39	0/3	W M 48	2/6

[a] Numerator = number of rabbits showing lesions, denominator = number of rabbits challenged

and St Louis 47 there was some lack of cross-protection, as there was also with strain S4 It should be noted, however, that in every instance where lesions occurred in the immune animals the incubation period of lesions was prolonged in comparison with that in the normal control animals, and the lesions were smaller than in the controls.

Viewing these results as a whole, there was good cross-protection with the Nichols strain in 14 of the 19 strains tested In 6 instances the cross-immunity tests were made by both of the procedures outlined above.

TABLE LI RESULTS OF CHALLENGE INOCULATION OF VARIOUS STRAINS OF SYPHILIS TREPONEMES IN RABBITS PREVIOUSLY INFECTED WITH THE NICHOLS STRAIN

Challenge strain	Results of challenge inoculation		Challenge strain	Results of challenge inoculation	
	Nichols immune [a]	Controls [a]		Nichols immune [a]	Controls [a]
Nichols	1/9	6/6	A G 39	0/6	4/4
S4 33	5/5	4/4	M S I	0/5	4/4
S6 33	2/5	4/4	L W 39	0/9	6/7
B T 37	1/8	7/7	M S II 39	0/5	4/4
N P 37	0/9	6/7	C J 39	0/5	4/4
L W 33	6/6	4/4	St Louis 47	5/6	4/4

[a] Numerator = number of rabbits showing lesions, denominator = number of rabbits challenged

The results with strain F.R.47 and W.M.48 were equivocal, while strains S4, L.W.38 and St Louis 47 showed evidence of a lower degree of cross-reactivity. Unfortunately, soon after these tests were completed most of the strains mentioned above were lost, so that it was not possible to secure more data of this nature on these particular strains. However, some confirmatory studies were made with the TPI test in respect of strain St Louis 47, as will be mentioned later (see page 225)

Since even those strains which failed to yield evidence of complete cross-protection with the method employed gave clear evidence of partial protection, it is difficult to know whether the differences are sufficiently great to warrant classifying them as belonging to one or more antigenic types, different from the group of which the Nichols strain is the prototype.

We may postulate the antigenic relationship of these strains on the following basis of the 19 syphilis strains, insufficient data are available on 2, 3 strains differ from the Nichols strain but give some degree of cross-protection, and 14 strains appear to be identical with the Nichols strain.

Cross-immunity tests on syphilis, yaws and cuniculi strains

An early paper, published from this laboratory, included data which suggested a high degree of cross-immunity between the particular strains of syphilis, yaws, and cuniculi treponemes employed in those experiments [15] Most of these studies involved the simultaneous challenge inoculation with the three species of treponemes, a technique later abandoned because of difficulties in the interpretation of results. Nevertheless, the data are not entirely meaningless and are re-presented here as a part of the entire picture

Experiment 1. Eighty rabbits of approximately the same age from a highly inbred Dutch Belt stock were divided into 4 groups of 20 each The groups were infected by the intratesticular route with *T. pallidum* (strain S6), *T. pertenue* (strain YC), and *T. cuniculi* (strain cuniculi B), respectively All rabbits developed lesions characteristic of the respective experimental disease. A fourth uninfected group was maintained under the same laboratory conditions.

Six months after the original inoculation all surviving animals, including the previously uninfected controls, were inoculated intracutaneously with normal testicular emulsion, or with one of three species of treponemes in each of the four quadrants of the back. The strains employed in the challenge inoculation were as follows. *T. pallidum*, Nichols strain, *T. pertenue*, strain YD-pre-1949; *T. cuniculi*, strain cuniculi A. It will be noted that these strains were all heterologous to the original infecting strains.

The results are shown in Table LII. It is evident that in general there was a substantial degree of cross-protection among these three species of treponemes. On the other hand there was clearly less cross-immunity

between the yaws and cuniculi strains

Experiment 2. In a second, somewhat similar, experiment one group of 19 rabbits was infected with strain cuniculi A and another group with strain cuniculi B Approximately 6 months later, after all the animals had progressed through typical infections, both groups were challenged, along with 15 normal rabbits, by the simultaneous intracutaneous inoculation of 2 strains of syphilis treponemes, Nichols and C J 39, a yaws strain YD, and a cuniculi strain cuniculi A The inoculum in this instance was frozen material in which it was difficult to estimate the number of viable treponemes; from the known effect of the size of the inoculum on the incubation period it can be estimated that the number of treponemes inoculated was probably of the order of 5000. Each strain was inoculated at 6 sites on the rabbits' back.

The results of these inoculations are shown in Tables LIII and LIV. In this experiment it is again evident that while cuniculi infection with two different strains induced in rabbits substantial degrees of cross-protection to challenge inoculation of syphilis treponemes this immunity was not of as high an order as might have been expected to occur with most syphilis strains It seems justifiable to conclude that these cuniculi strains do differ somewhat—at least in antigenic structure—from the two syphilis strains tested

Experiment 3 Ten rabbits were inoculated intratesticularly with the Nichols strain of *T pallidum* All developed typical syphilitic orchitis. Four months after the first infection these animals together with 8 control rabbits were inoculated intracutaneously on the back with yaws treponemes of strain YA, and the homologous strain (Nichols) of syphilis treponemes The results are shown in Table LV

In these tests the syphilis animals showed good cross-protection against the same strain of syphilis treponemes, but incomplete protection against the YA strain of yaws treponemes

Experiment 4 One group of rabbits was infected by intratesticular inoculation with strain cuniculi A, and another group with syphilis strain C J. 39 All rabbits showed lesions regarded as characteristic of the respective infections Eight months after the original inoculation the surviving animals, along with a group of normal animals which had been maintained as controls and a group of newly acquired normal rabbits, were challenged with the Nichols strain of syphilis treponemes. The challenge inoculation was made intracutaneously with 500 treponemes at each of 4 sites on the rabbits' back

The results with strain F.R.47 and W.M.48 were equivocal, while strains S4, L W.38 and St Louis 47 showed evidence of a lower degree of cross-reactivity Unfortunately, soon after these tests were completed most of the strains mentioned above were lost, so that it was not possible to secure more data of this nature on these particular strains. However, some confirmatory studies were made with the TPI test in respect of strain St Louis 47, as will be mentioned later (see page 225)

Since even those strains which failed to yield evidence of complete cross-protection with the method employed gave clear evidence of partial protection, it is difficult to know whether the differences are sufficiently great to warrant classifying them as belonging to one or more antigenic types, different from the group of which the Nichols strain is the prototype.

We may postulate the antigenic relationship of these strains on the following basis of the 19 syphilis strains, insufficient data are available on 2; 3 strains differ from the Nichols strain but give some degree of cross-protection, and 14 strains appear to be identical with the Nichols strain.

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Experiment 1 Eighty rabbits of approximately the same age from a highly inbred Dutch Belt stock were divided into 4 groups of 20 each. The groups were infected by the intratesticular route with *T. pallidum* (strain S6), *T pertenue* (strain YC), and *T cuniculi* (strain cuniculi B), respectively All rabbits developed lesions characteristic of the respective experimental disease A fourth uninfected group was maintained under the same laboratory conditions.

Six months after the original inoculation all surviving animals, including the previously uninfected controls, were inoculated intracutaneously with normal testicular emulsion, or with one of three species of treponemes in each of the four quadrants of the back The strains employed in the challenge inoculation were as follows: *T. pallidum*, Nichols strain, *T. pertenue*, strain YD-pre-1949; *T. cuniculi*, strain cuniculi A. It will be noted that these strains were all heterologous to the original infecting strains.

The results are shown in Table LII. It is evident that in general there was a substantial degree of cross-protection among these three species of treponemes. On the other hand there was clearly less cross-immunity

TABLE LV IMMUNITY OF RABBITS INFECTED WITH SYPHILIS TREPONEMES TO CHALLENGE INOCULATION WITH YAWS TREPONEMES AND HOMOLOGOUS SYPHILIS TREPONEMES

First infection	Results of challenge inoculation (28 days)	
	Syphilis (Nichols) [a]	Yaws (YA) [a]
Syphilis (Nichols strain)	0/10	6/10
None (controls)	8/8	8/8

[a] Numerator = number of animals showing lesions, denominator = number of animals challenged

The results, which are shown in Table LVI, confirm those presented in the preceding tables in that again complete protection was demonstrated between the two syphilis strains, but only partial protection between the cuniculi A strain and the Nichols strain

The only difference noted between the old controls and the new controls was a slight but definite prolongation of the incubation period of lesions in the latter group By the 21st day after inoculation only 1 of 7 old controls had shown no lesions, while 5 of the 8 new controls had no lesions This could probably be explained by the presence of mild intercurrent infections with slight elevation of body temperature in the animals more recently brought into the laboratory

On the basis of these data we may again attempt a rough antigenic characterization of the strains studied here In agreement with the earlier studies, mentioned in the preceding section, the strains S6, C J 39 and the Nichols strain were found to be antigenically similar to the Nichols strain

The data relative to the yaws strains are less clear Strain YA did not show good cross-protection with the Nichols strain (Table LV)

TABLE LVI IMMUNITY OF RABBITS INFECTED WITH CUNICULI AND SYPHILIS TREPONEMES TO CHALLENGE INOCULATION WITH A HETEROLOGOUS STRAIN OF SYPHILIS

First infection	Number challenged	Size of lesions 35 days after challenge inoculation with Nichols strain		
		None	+, ++	+++ ++++
Cuniculi A	13	4	7	2
Syphilis (C J 39)	9	9	0	0
8 month controls	7	0	0	7
New controls	8	0	1	7

TABLE LII CROSS-IMMUNITY IN TREPONEMAL INFECTIONS FIRST INFECTION, INTRATESTICULAR, CHALLENGE INOCULATION 6 MONTHS LATER

First infection	Results of challenge inoculation with indicated strain		
	Syphilis (Nichols) [a]	Yaws (YD-post-1949) [a]	Cuniculi (cuniculi A) [a]
Syphilis - S6 33	2/18	0/18	4/18
Yaws - YC	3/17	0/17	10/17
Cuniculi — cuniculi B	11/18	4/18	0/18
None (controls)	18/18	18/18	18/18

[a] Numerator = number of animals showing lesions, denominator = number of animals challenged

TABLE LIII CROSS-IMMUNITY TO OTHER TREPONEMAL INFECTIONS OF RABBITS INFECTED WITH CUNICULI TREPONEMES

First infection	Results of challenge inoculation			
	Syphilis (Nichols) [a]	Syphilis (C J 39) [a]	Yaws (YD-pre 1949) [a]	Cuniculi (cuniculi A) [a]
Cuniculi A	15/19	8/19	11/19	0/19
Cuniculi B	17/21	12/21	14/21	0/21
None (controls)	15/15	15/15	9/15	2/15

[a] Numerator = number of animals showing lesions, denominator = number of animals challenged

TABLE LIV COMPARATIVE SIZE OF LESIONS FOLLOWING CHALLENGE INOCULATION OF SYPHILIS TREPONEMES

First infection	Number inoculated	Number of rabbits showing lesions of indicated size					
		Nichols strain			C J 39 strain		
		Number of lesions	+, ++	+++, +++	Number of lesions	+ ++	+++, ++++
Cuniculi A	19	4	11	4	11	6	2
Cuniculi B	21	4	12	5	9	12	0
None (controls)	15	0	0	15	0	2	13

ciates, in which similar types of studies were made in monkeys. These investigators, on the basis of cross-immunity studies, have likewise recognized a distinction between strains of syphilis and yaws treponemes

Cross-immunity between newly isolated strains

In studying cross-immunity phenomena among the newly isolated strains in this laboratory we have adopted the simplest method available, that of invoking immunity in rabbits by experimentally infecting them with a "standard" strain, the Nichols strain of *T. pallidum*, then challenging groups of these animals with one or another of the new strains

The initial inoculation of the Nichols strain was always intratesticular and with a large dose of treponemes Challenge inoculation, which was made between 3 and 4 months after the original inoculation, was always intracutaneous, at 4 sites on the shaved back of the animal, with a dose of 5000 treponemes at each site. Control animals were routinely inoculated with the same suspension and the same dose of treponemes, test animals

TABLE LVII CROSS-IMMUNITY IN TREPONEMAL INFECTIONS RESULTS OF CHALLENGE INOCULATION OF NICHOLS-IMMUNE RABBITS WITH VARIOUS NEWLY ISOLATED STRAINS

Challenge strain		Results of challenge inoculation	
Group	Strain	Immune rabbits [a]	Normal rabbits [a]
Syphilis	Chicago	0/7	8/8
"	Baghdad A	0/6	7/7
"	Baghdad B	0/4	4/4
"	Mexico	0/4	4/4
Yaws	Haiti A	3/7	8/8
"	Haiti B	5/8	8/8
"	Indonesia B	0/4	3/4
"	Samoa D	2/8	8/8
"	Samoa F	0/4	4/4
Bejel	Syria A	3/7	6/7
"	Syria B	2/7	7/7
"	Iraq B	8/17	16/16
Endemic syphilis	Bosnia A	0/4	4/4
"	Bosnia B	0/4	4/4
Dichuchwa	Bechuanaland C	0/4	4/4
"	Bechuanaland D	0/3	4/4

[a] Numerator = number of rabbits showing lesions, denominator = number of rabbits inoculated

Strain YC showed cross-protection with the Nichols strain, and strain YD-pre-1949 showed good cross-protection with syphilis strain S6, as well as with strain YC (Table LII). It will be noted below that McLeod & Magnuson [9] found in one experiment rather poor cross-protection between our YC strain and the Nichols strain.

Clearly, however, both of the two cuniculi strains tested showed a relatively poor degree of cross-immunity with Nichols, S6, and C J. 39 syphilis strains, and with YC and YD-pre-1949 yaws strains (Tables LII, LIII, LIV and LVI)

Viewed from a different angle, these experiments provide ample evidence that there is some degree of cross-immunity among all the treponemal strains tested. These data should be reviewed in conjunction with those derived from serological tests as presented later

Studies in other laboratories. McLeod & Magnuson [9] extended the studies referred to above to include determination of asymptomatic reinfection as well as symptomatic reinfection. The Nichols syphilis strain and our YC strain were used. The first infection was by the intrastesticular route and the challenge inoculation was intradermal. Curative penicillin treatment was given 7-10 months after first infection Graded challenge doses were employed. Rabbits remaining asymptomatic for 4 months were subjected to lymph-node transfer.

In the group of rabbits originally infected with syphilis, 23 were reinoculated with the homologous strain. Of these, none developed specific lesions, and in only 8 were lymph nodes positive. By contrast, of 22 syphilis rabbits challenged with yaws strain YC, 10 developed yaws lesions, one was shown to have asymptomatic infection, while in 11 lymph-node transfer was negative.

In the group of rabbits originally infected with yaws, 24 were reinoculated with the homologous strain of yaws treponemes. Of these, none developed yaws lesions, and only one showed evidence of asymptomatic infection. Again, by contrast, of 28 yaws rabbits challenged with the Nichols strain of syphilis treponemes, 21 developed syphilitic lesions, and 7 seemed to be solidly immune.

While the results of those experiments differ in detail from those reported from the authors' laboratory, the over-all picture is essentially the same. There is definitely some degree of cross-immunity between yaws and syphilis, but not as much as between strains of the same species. In general the syphilis treponeme, or at least the Nichols strain, appears to be either a more efficient antigen, or to have a broader antigenic spectrum, than the yaws group of strains thus far tested

Reference should be made here to the investigations of Matsumoto [12] and his associates, in which cross-immunity studies between yaws and syphilis were made in rabbits, and to the studies of Schöbl [14] and his asso-

ciates, in which similar types of studies were made in monkeys. These investigators, on the basis of cross-immunity studies, have likewise recognized a distinction between strains of syphilis and yaws treponemes.

Cross-immunity between newly isolated strains

In studying cross-immunity phenomena among the newly isolated strains in this laboratory we have adopted the simplest method available, that of invoking immunity in rabbits by experimentally infecting them with a "standard" strain, the Nichols strain of *T. pallidum*, then challenging groups of these animals with one or another of the new strains.

The initial inoculation of the Nichols strain was always intratesticular and with a large dose of treponemes. Challenge inoculation, which was made between 3 and 4 months after the original inoculation, was always intracutaneous, at 4 sites on the shaved back of the animal, with a dose of 5000 treponemes at each site. Control animals were routinely inoculated with the same suspension and the same dose of treponemes; test animals

TABLE LVII CROSS-IMMUNITY IN TREPONEMAL INFECTIONS RESULTS OF CHALLENGE INOCULATION OF NICHOLS-IMMUNE RABBITS WITH VARIOUS NEWLY ISOLATED STRAINS

Challenge strain		Results of challenge inoculation	
Group	Strain	Immune rabbits [a]	Normal rabbits [a]
Syphilis	Chicago	0/7	8/8
"	Baghdad A	0 6	7/7
"	Baghdad B	0/4	4/4
"	Mexico	0/4	4/4
Yaws	Haiti A	3/7	8/8
"	Haiti B	5/8	8/8
"	Indonesia B	0/4	3/4
"	Samoa D	2/8	8/8
"	Samoa F	0/4	4/4
Bejel	Syria A	3/7	6/7
"	Syria B	2/7	7/7
"	Iraq B	8/17	16/16
Endemic syphilis	Bosnia A	0/4	4/4
"	Bosnia B	0/4	4/4
Dichuchwa	Bechuanaland C	0/4	4/4
	Bechuanaland D	0/3	4/4

[a] Numerator = number of rabbits showing lesions, denominator = number of rabbits inoculated

that remained negative were observed for 4 weeks after the appearance of lesions in the controls.

The results of these cross-immunity studies are shown in Table LVII. It will be noted that the Nichols strain gave a high order of cross-immunity to all the new strains isolated from patients with venereally acquired syphilis, namely, the Chicago, Baghdad A, Baghdad B and Mexico strains.

The protection afforded by the Nichols strain against the yaws strains as a group was not nearly so high as against the syphilis strains, although there was abundant evidence of substantial cross-protection. There was likewise less cross-protection with the bejel strains, whereas a high order of immunity was afforded against the two endemic syphilis strains, Bosnia A and Bosnia B, and against the two dichuchwa strains, Bechuanaland C and Bechuanaland D.

In most of these cross-immunity experiments we are dealing with small numbers of animals in any one series. Doubtless the sampling error is high. When reviewed as a group, however, the data become more meaningful, the yaws strains as a group and the bejel strains as a group show significantly lower orders of cross-immunity than do the syphilis strains, the endemic syphilis strains and the dichuchwa strains.

Evidence Obtained From the Study of Immobilizing Antibody

Discovery of immobilizing antibody and the development of the TPI test raised hopes that this might provide a definitive tool for the study of antigenic relationship within the *Treponema* group of organisms. However, meaningful studies require quantitative determination and, unfortunately, technical difficulties have limited the utilization of the quantitative test in the study of the problem, although informative data have been acquired

Since there is great variability in the TPI test from day to day and from one laboratory to another, not only is it necessary to include standard serum pools and a reference treponeme strain in every series of tests, but each series must be repeated, preferably several times, in order to be beyond the limits of normal variation. With those points in mind, comparative antigenic analysis can be made principally in two ways: one by testing a standard pool of serum prepared from animals infected with one or another species of treponeme against each "*unknown*" *strain of treponeme*; and the other, by testing serum from man or animals infected with different strains of treponemes against a few selected strains of treponemal organisms.

The first approach is the most direct and theoretically the best, but it embodies the largely insuperable difficulty that many strains of treponemes, particularly those belonging to the yaws group, cannot be brought to the point where they would produce testicular lesions in rabbits which would

provide an adequate source of treponemes for the TPI test. Only meager data have therefore been collected by this method

The second approach is more practicable, but the accumulation of reliable data is laborious. A few strains of treponemes, each believed to be representative of a particular syndrome, are selected to provide a source of treponemes for the TPI test Sera from many sources can then be tested against these representative strains, and some notion can be derived as to their antibody pattern by study of comparative titers For example, if serum from a rabbit experimentally infected with a particular treponeme strain consistently shows a low titer of immobilizing antibody to a representative syphilis strain, and a high titer to a selected yaws strain, it can be inferred that the strain being tested is more closely related to the yaws strain than to the syphilis strain.

With the foregoing principles in mind we shall now examine the relatively limited data bearing on this subject The first study of this nature was that reported by Khan, Nelson & Turner [a] in which 7 strains of treponemes were investigated and the essentials of both the above-mentioned methods were used. Included in the study were syphilis strains Nichols, S6, M.S I. and St Louis; yaws strains YA and YD, and cuniculi strain cuniculi A.

To obtain data on the reproducibility of the quantitative immobilization test, a single specimen from a rabbit infected with each of the strains of treponeme in the study was tested several times against the homologous strain. The results, presented in Table LVIII, show a maximum variation of less than a threefold serum dilution, and, if anything, represent better

TABLE LVIII HOMOLOGOUS IMMOBILIZING TITER OF VARIOUS ANTISERA REPEATED ON SEVERAL OCCASIONS DEMONSTRATING DEGREE OF REPRODUCIBILITY OF TITRATION TECHNIQUE *

Group	Strain	Serum	Titers obtained on different tests			
Syphilis	Nichols	Pool No 8	700	540	600	50
"		"	600	810	580	700
	"	"	640	500	600	1100
	St Louis	1125	470	700	490	700
"	S6	1183	1150	2000	1450	
"	M S I	1341	780	650	790	
Yaws	YA	1129	3050	3300	3050	
	YD post 1949	1103	100	90	70	220
Cuniculi	CA	1064	500	520	720	

* From Khan, Nelson & Turner [a]

reproducibility than is to be anticipated in the general run of quantitative TPI tests

A group of rabbits was then infected with each of three syphilis strains—S6, M S I. and St Louis—and two further groups were infected with the Nichols strain. Sera from the first group of animals infected with the Nichols strain were collected from 4 to 7 months after infection and tested as a single pool. Sera from the second group of Nichols-strain animals were collected 6 months after infection, and sera from the groups infected with syphilis strain S6, M S I and St Louis, respectively, were collected at 4 months All serum specimens except the Nichols pool were tested as individual specimens, and the titer of immobilizing antibody for all the sera from a particular group is expressed as the arithmetic mean in Table LIX, which has been adapted from Tables 9, 10, 11 and 12 of the paper by Khan, Nelson & Turner [6]

TABLE LIX IMMOBILIZING ANTIBODY TITERS OF ANTISERA FROM RABBITS INFECTED WITH VARIOUS STRAINS OF SYPHILIS TREPONEMES AGAINST THE SAME GROUP OF HOMOLOGOUS AND HETEROLOGOUS STRAINS * †

Antisera	Number of sera in group	Mean titer indicated strain of treponemes as antigen			
		Nichols	S6	M S I	St Louis
Nichols pool	7	600	360	740	400
Nichols	4	832	682	62C	378
S6	6	1383	1445	1476	966
M S I	6	191	225	356	190
St Louis	5	171	94	164	1272

* Computed as arithmetic means of the dilution giving immobilization of 50 % of treponemes in the test

† Adapted from Khan, Nelson & Turner [6]

Inspection of Table LIX reveals that there is scarcely more than a two-fold difference in the mean titers of these sera against the four strains of syphilis treponemes, except in the case of the sera from the group of animals infected with the St Louis strain In the latter instance the mean titer is significantly higher when tested against the homologous strain in comparison with the titers against the other syphilis strains; analysis of the readings for the individual sera shows that each of the five specimens gave substantially higher titers against treponemes of the St Louis strain than against Nichols, S6 or M S.I. treponemes Actually the mean titers of the sera from the other groups of animals are somewhat lower against

the St Louis strains than against the others tested, but the differences are not great and may not be significant.

It will be recalled from the data presented in Tables L and LI (page 217), that the St Louis strain of syphilis treponemes was one of the strains tested that showed the poorest cross-protection with the Nichols strain Therefore it may be justifiable to conclude that antigenically this strain does differ somewhat from the others tested, we regard the evidence as insufficient, however, to state categorically that the St Louis strain should be regarded as belonging to a different antigenic type.

As a part of this same study Khan, Nelson & Turner [6] carried out cross-immobilization tests on the Nichols syphilis strain, the YA and YD-post-1949 yaws strains and the cuniculi A strains of cuniculi treponemes Groups of animals were infected as in the preceding experiment, the Nichols animals being those to which reference was made in Table LIX Serum specimens from the YA animals were collected 6 months after infection, specimens from cuniculi A animals at 4 months, while from YD-post-1949 animals sera were collected at 2-6 months

TABLE LX IMMOBILIZING ANTIBODY TITERS OF ANTISERA FROM SYPHILIS, YAWS AND CUNICULI RABBITS AGAINST HOMOLOGOUS AND HETEROLOGOUS STRAINS OF TREPONEMES * †

Antisera	Number of sera in group	Mean titer with indicated strain of treponeme as antigen			
		Nichols	YA	YD post 1949	Cuniculi A
Syphilis (Nichols Pool I)	7	600	500	800	30
Syphilis (Nichols)	4	832	415	445	39
Yaws (YA)	4	710	1420	477	32
Yaws (YD post-1949)	6	503	883	682	28
Cuniculi (cuniculi A)	4	35	47	58	492

* Computed as arithmetic means of the dilution giving immobilization of 50% of treponemes in the test

† Adapted from Khan, Nelson & Turner [6]

Sera, except for those in the Nichols pool, were titered separately, and the arithmetic means of the titers are shown in Table LX, which has been adapted from Tables 5, 6, 7 and 8 of the paper by Khan, Nelson & Turner.[6] It will be observed that there are no significant differences in titer for the sera from Nichols, YA and YD animals against each of those strains of treponemes. However, a significant difference in the titers for these sera against the cuniculi A strain is noted, and likewise sera from the rabbits infected with cuniculi A strain gave consistently lower titers against the Nichols strain of syphilis treponemes and the YA and YD strain of yaws organisms.

These latter results appear to be sufficiently consistent to justify the opinion that the cuniculi A strain does differ antigenically from the syphilis and yaws strains tested.

Magnuson, Thompson & McLeod[10] studied cross-immobilization patterns for 3 strains of syphilis treponemes—the Nichols strain, and their own strains, 20A and 20B Their TPI tests were made on a qualitative, rather than on a quantitative, basis and titers of the sera tested were determined in terms of the percentage of rabbits infected with each strain that showed a positive TPI test. With this indirect method no differences were noted among the three strains.

Cross-immobilization tests on newly isolated strains

The treponemal strains studied by Khan, Nelson & Turner[6] were ones which had been isolated a number of years previously and propagated through many serial passages in rabbits. The question could therefore justifiably be raised as to whether their antigenic pattern might not have become altered in the period since their isolation from man, their natural host. Consequently, it seemed desirable to study newly isolated strains by means of cross-immobilization tests and cross-agglutination tests.

The method of procedure was essentially as follows. Rabbits were infected with one or another of the strains to be tested. When the titer of specific antibody was presumed to have reached a high level, commonly about the 4th month after infection, the animals were bled, and the sera tested in threefold dilutions for immobilizing antibody against several strains of treponemes selected to represent various treponemal syndromes, such as syphilis, yaws, bejel, and cuniculi infection.

The results of these quantitative immobilization tests carried out by Miss Nell and Dr Hardy are shown in Table LXI Titers are expressed in terms of the reciprocal of the dilutions calculated to immobilize 50% of the test organisms

In analysing the results presented in Table LXI, it is apparent first that wide fluctuations in titer occur from test to test, even when the same serum and the same treponeme strain are used. Since all of these tests were made by experienced personnel, we are forced to the conclusion that there is inevitably a wide variation in readings due to technical factors alone, and that the interpretation of quantitative TPI titers must take due account of this fact

treponemes than in those with bejel or cuniculi treponemes, but we are hesitant in regarding this as necessarily significant.

TABLE LXI. TITERS OF IMMOBILIZING ANTIBODY OF SERA FROM RABBITS INFECTED WITH VARIOUS STRAINS OF TREPONEMES

Infecting strain of treponeme	Rabbit number source of test serum	Strain of treponeme used as antigen in immobilization test with indicated titer				
		Syphilis		Yaws	Bejel	Cuniculi
		Nichols	Chicago	Haiti B	Iraq B	Cuniculi A
Nichols	Nichols pool	270 520 385	850 700 620	650 410 265 130	50 30	190 < 50
Baghdad A	43 90	37 130	47	16	14 19	13 85 < 10
Baghdad A	43 91	60	49	200 42		
Mexico A	46-81	43 200		< 10	26 27	13 18 < 10
Baghdad B	44 12	36		190 41		
Bosnia A	44-18	80 150	73	< 10	82 75	17 41 < 10
Syria A	44 30	23 23	52	19	12 5 17	15 < 10 < 10
Syria B	44 11	46 100	13	14	24 17	13 22 17
Haiti A	46 12	34 30		< 10	10 16	< 10 10 < 10
Indonesia B	46-04	21 72		13	23 23	10 55 110
Indonesia B	44 37		19	25 < 10 < 10		
Cuniculi A	44-09	11	10	59 < 10 < 10	< 10 < 10	48 64 65

These latter results appear to be sufficiently consistent to justify the opinion that the cuniculi A strain does differ antigenically from the syphilis and yaws strains tested.

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In analysing the results presented in Table LXI, it is apparent first that wide fluctuations in titer occur from test to test, even when the same serum and the same treponeme strain are used. Since all of these tests were made by experienced personnel, we are forced to the conclusion that there is inevitably a wide variation in readings due to technical factors alone, and that the interpretation of quantitative TPI titers must take due account of this fact

treponemes than in those with bejel or cuniculi treponemes, but we are hesitant in regarding this as necessarily significant.

TABLE LXII RESULTS OF QUANTITATIVE TREPONEMAL AGGLUTINATION TESTS ON SERUM FROM RABBITS INFECTED WITH ONE OF A NUMBER OF NEWLY ISOLATED STRAINS

Serum tested			Titer [a] against antigen prepared from indicated strain				VDRL titer on unabsorbed sera
Group	Strain	Rabbit number	Nichols	Haiti B	Iraq B	Cuniculi A	
Syphilis	Chicago	43 94	1280 640	1280 640	320 640	320 160	32
"	Baghdad A	43 90	320 320	160 320	320 1290	80	32
"	Baghdad B	44 13	160 160	160 160	160	0	16
"	Mexico A	46-81	320 640	320 320	2560 2560	320	16
Endemic syphilis	Bosnia A	44 18	320 320	320 320	640 640	160 80	18
"	Bosnia B	44 22	320 320	640 640	640	160	16
Bejel	Syria A	44 30	640 320	160 160	1280	80	32
"	Syria B	44 11	640 640	160	1290 2560	80	16
"	Iraq B	44 32	640 320	320 640	1290 640	80	2
Yaws	YD post 1949	43 [illegible]2	640 640	320 640	5120	320	32
"	Haiti A	46-12	1280 640	160 160	1280	40	8
"	Haiti B	44-34	1280 640	1290 2560	1280 1280	320 320	64
"	Indonesia B	46-04	640 640	640 2560	1280 2560	640 1280	64
	Samoa D	51 39	1280 1280	640 1280	640 1280	80	0
"	Samoa F	51-68	640 320	160 320	640	160	—
Cuniculi	Cuniculi A	49-41	320 320	1280 2560	1280	640 1280	128
"	Cuniculi A	49-42	1280 1280	1280 1280	2560 1280	640 1280	64

[a] Titer = reciprocal of the final dilution of serum in the test

As a generalization, it can also be stated that sera from syphilis, yaws, and bejel animals gave lower titers with cuniculi treponemes than with those from other species, while serum from one cuniculi animal gave higher titers with the homologous strain of treponeme than with the others tested. Again, however, we cannot regard these results as definitive.

Certainly, among the syphilis, yaws and bejel group there is no indication of a clear-cut species difference in the antigen-antibody system active in the TPI test. Syphilis sera, for example, immobilized both syphilis and yaws treponemes in about the same titer, and although a strain of bejel treponemes gave lower titer with Nichols antisera, it does not appear that there were significant or consistent differences.

This is all very disappointing, largely because of the unsatisfactory technical status of the quantitative TPI test And yet the over-all picture established here in rough outline corresponds to that which emerges from the use of the treponemal agglutination test, as described below, and indeed to that obtained from the cross-immunity tests referred to earlier.

Cross-agglutination tests on newly isolated strains

In Table LXII are shown the results of quantitative treponemal agglutination tests performed by Miss Nell and Dr Hardy on serum from rabbits infected with one of a number of newly isolated strains. Serum specimens were collected from each animal approximately 4 months after infection As antigen 4 representative strains were selected· the Nichols strains of syphilis, the Haiti B strain of yaws, the Iraq B strain of bejel, and the cuniculi A strain of cuniculi treponemes In most instances each serum was titrated twice against the same antigen All sera were absorbed with cardiolipin antigen before agglutination tests were performed. All readings were made without knowledge of the particular serum under test. For comparative purposes titers of the unabsorbed sera against VDRL antigen are also shown in the table

Analysis of these data permit several conclusions. First, on the whole the titers of a given serum have a fairly good degree of reproducibility with rarely more than twofold variation. Second, no consistent pattern is apparent with respect to comparative titers of sera from animals infected with strains of treponemes from a particular clinical syndrome; in general the titers obtained with the cuniculi antigen tended to be lower with all sera, with the possible exception of the sera from cuniculi rabbits. Third, the results suggest that various pools of antigen vary in agglutinability; in this series the Iraq B antigen tended to agglutinate in higher titer with all sera than did the other antigens. Fourth, there was no correlation between the VDRL titers of unabsorbed sera and the agglutination titers of absorbed sera.

In short, these data provide no evidence of the existence of antigenic differences among the various strains and species of treponemes that can

including the controls were challenged intracutaneously with a dose of 200 *T. pallidum* at each of 4 sites on the back Among the animals of the control group the mean incubation period of the syphilitic lesions was slightly shorter than that in the other two groups (Group I, 22 5±2.7 days; Group II 27 0±2.51 days; Group III, 27 5±3 7 days) The lesions in the animals of Group III tended to remain slightly smaller than those in the animals of Groups I and II. The animals in Groups II and III did not develop treponemal immobilizing antibodies as a result of the injections of Reiter organisms.

These results suggest a very low grade cross-immunity, which was slight at best and may have been due to some non-specific stimulation of resistance In retrospect it would have been preferable if the control animals had received injections of an antigenically unrelated material Other investigators have likewise failed to demonstrate significant degrees of cross-immunity,[2, 7, 8 11] although the quantitative aspects of those experiments were not perhaps as carefully controlled as were those of Gelperin

In addition to cross-immunity studies, Gelperin [5] prepared three fractions from a tryptic digest of washed cultures of Reiter organisms These investigations together with the more recent fractionation studies by D'Alessandro & Dardanoni [3] and Puccinelli [13] have already been discussed in Chapter 5. Gelperin found slight serologic activity in two fractions when tests were made with normal rabbit and human sera, and a somewhat greater activity with syphilitic rabbit and human sera The antibody was distinct from Wassermann antibody, a finding which was in agreement with earlier studies of Beck [1] and Kolmer.[8] The Italian investigators,[3, 13] however, appear to have worked with a Reiter strain which produced Wassermann antibody. Rabbit antiserum prepared against other strains of cultured treponemes—Kazan, Nichols, and S26—reacted with Gelperin's fraction K, thus indicating a close relationship among these various strains, as previously noted by Eagle & Germuth [4]

The isolation of highly specific complement-fixing antigens from cultured treponemes described by the Italian investigators (Chapter 5) has reopened the question of the relationship of the cultivated strains to the pathogenic treponemes. Perhaps they may be closer than has generally been supposed.

REFERENCES

1 Beck, A (1939) The role of the spirochete in the Wassermann reaction, *J Hyg (Lond)*, 39, 398

2 Bessemans, A & De Geest, B (1930) Contribution à l'etude du *Treponema pallidum* Aristowsky-Hoeltzer Pouvoir pathogène et antigenique, immunite, *C R. Soc Biol (Paris)*, 103, 522

3 D'Alessandro, G. & Dardanoni, L. (1953) Isolation and purification of the protein antigen of the Reiter treponeme, *Amer J Syph*, 37, 137

be detected by the treponemal agglutination test as now performed in this laboratory. (The results with the cuniculi antigen may be interpreted as a possible exception to this statement.) It will be recognized that essentially the same conclusions were reached concerning strains of syphilis, yaws and bejel treponemes by cross-immunity studies and by studies of the patterns of immobilizing antibodies.

Antigenic Relationship between the Reiter and Related Treponemes and Pathogenic Species

Ever since the isolation in pure culture of spirochetes which morphologically resemble *T. pallidum* and other pathogenic treponemes, hope has been sustained that these organisms might be sufficiently closely related to the pathogenic variety to serve in some diagnostic capacity. In general these hopes have not been fulfilled (see Chapter 5). Numerous antigens of one sort or another have been put on the market, but in most cases they have offered no biological or practical advantage over standard lipoidal antigens

Immunologic Studies of the Reiter Treponeme

Gelperin [5] in our laboratory investigated certain immunologic properties of the Reiter spirochete, with particular reference to its possible relationship to syphilis treponemes and related organisms One aspect of the study was the intensive " immunization " of rabbits with large doses of Reiter organisms and subsequent challenge of these animals with virulent *T. pallidum*

Three groups of 10 rabbits each were established and a serum specimen obtained from each animal Group I was maintained as a control and received no injections. The animals of Group II were injected intravenously and intraperitoneally with a saline suspension of Reiter spirochetes adjusted to a spirochetal nitrogen content of 50 μg per ml Initially injections were given intravenously three times a week for 11 weeks. Since 5 animals of this group died, presumably from pulmonary emboli or from anaphylactic shock, later injections were given intraperitoneally.

Group III animals received concentrated spirochetes in an adjuvant mixture containing Falba oil and a suspension of *Mycobacterium phlei* Injections were given subcutaneously every other week, the spirochetal nitrogen content of the mixture being increased from 50 μg per week to 500 μg. The animals of two groups may therefore by regarded as receiving a major antigenic stimulus. Post-injection specific agglutination titers varied from 1 : 128 to 1 : 2048, titers being about the same in both Group II and Group III. It is interesting that no animal developed a significant titer of Wassermann antibody.

Approximately 2 weeks after the last " immunizing " injection, or approximately 3 months after the initiation of the series, all the animals

Chapter 9

COMPARATIVE SUSCEPTIBILITY OF STRAINS OF TREPONEMES TO PENICILLIN

One of the important practical questions in the field of treponematosis control is whether all species and strains of pathogenic treponemes are equally susceptible to antibiotics, particularly penicillin; whether one scheme of treatment developed for, and laboriously tested in, one of the clinical syndromes of the treponematosis group of diseases, as it occurs in one locality, may reasonably be expected to be equally effective in other localities and against other clinical syndromes

In addition to this very practical question, there is a more theoretical problem concerned with the relation of one strain of treponeme to another. In other words Are there significant similarities or differences in the susceptibility of various strains of treponemes to penicillin that may serve as an index to the biological relationships existing within the family of treponemal organisms?

With the foregoing considerations in mind, studies have been made of the comparative susceptibility of most of our newly isolated strains to penicillin G.

Test Procedures

Tests for penicillin sensitivity have been made by both the *in vivo* and the *in vitro* methods described in Chapter 6 The *in vivo* method was developed in this laboratory by Turner, Cumberland & Li [2] The *in vitro* method follows, in principle, procedures used for other organisms, but was adapted as a practical procedure for treponemes only after painstaking experimentation by Nell,[1] working in this laboratory.

Results of "In Vivo" Tests

A rather wide experience with the Nichols strain has established that a dosage level of 0 25 mg of crystalline penicillin G per kg of body-weight will, in a high proportion of rabbits, reduce the treponeme count from 200 or

4. Eagle, H & Germuth, F (1948) Serologic relationships between five cultured strains of supposed *T pallidum* (Noguchi, Nichols, Kroo, Reiter and Kazan) and two strains of mouth treponemata, *J. Immunol*, 60, 223
5. Gelperin, A (1951) Immunochemical studies of the Reiter spirochete, *Amer. J Syph*, 35, 1
6. Khan, A S, Nelson, R A, jr & Turner, T. B (1951) Immunological relationships among species and strains of virulent treponemes as determined with the treponemal immobilization test, *Amer J. Hyg*, 53, 296
7 Kolmer, J A (1930) Failure of vaccination of rabbits against syphilis, with a note on selective localization of *Spirochaeta pallidum*, *Amer. J. Syph.*, 14, 236
8 Kolmer, J A, Kast, C C & Lynch, E R (1941) Studies on the role of *Spirochaeta pallida* in the Wassermann reaction. II The relation of spirochetal antibodies to the Wassermann reagin, *Amer. J Syph*, 25, 412
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10 Magnuson, H J, Thompson, F. A & McLeod, C. P (1951) Relationship between treponemal immobilizing antibodies and acquired immunity in experimental syphilis, *J Immunol*, 35, 146
11. Mason, H C (1939) Avirulence of culture *Spirochaeta pallida*, *Urol cutan Rev*, 43, 733
12. Matsumoto, S (1930) *Experimental syphilis and framboesia*, Kioto (*Monographiae Actorum Dermatologicorum*, Series B, *Syphilidologica*, No. 3)
13 Puccinelli, V A (1952) Plurality of antibodies in syphilitic serum and clinical practice, *Brit J vener Dis*, 28, 184
14 Schöbl, O (1930) Immunologic reciprocity between syphilis and yaws, *Philipp. J. Sci*, 43, 583
15 Turner, T B & McLeod, C. P (1942) Cross immunity in experimental syphilis, yaws and venereal spirochetosis of rabbits, *Trans. Ass Amer Phys*, 57, 265

It is evident that these data are far from definitive. Taken as a whole, however, they suggest that most of the newly isolated strains have the same degree of penicillin sensitivity as the Nichols strain

Results of " In Vitro " Tests

The results of *in vitro* tests of penicillin sensitivity on newly isolated strains, recorded in Table LXIV, *are those reported by Nell*[1] *from this* laboratory. The sensitivity is expressed in terms of the concentration of penicillin G that will immobilize 50% of the treponemes in the suspension (IC_{50}) during 18 hours' incubation at 35°C. The concentrations of penicillin are expressed in μg/ml.

TABLE LXIV COMPARATIVE "IN VITRO" SENSITIVITY OF NEWLY ISOLATED STRAINS OF TREPONEMES TO PENICILLIN *

Group	Strain	IC_{50} [a] after 18 hours incubation at 35°C Individual assay – μg/ml	Mean
Syphilis	Nichols	0.002, 0.0011 0.0018 0.002 0.007 0.0015, 0.0021 0.0021 0.0031 0.0022 0.0025	0.002
"	Chicago	0.0011 0.0017	0.0014
	Baghdad A	0.0012, 0.0083 0.0021, 0.0048	0.0041
"	Baghdad B	0.001, 0.0037 0.0048	0.0032
Yaws	Haiti A	0.0011 0.0021	0.0016
"	Haiti B	0.0008 0.0035 0.0039	0.0027
Bejel	*Syria A*	*0.002, 0.0016, 0.001, 0.0043*	*0.0022*
"	Syria B	0.0019, 0.0023	0.0021
"	Iraq B	0.0032, 0.001 0.002 0.0024	0.0021
Endemic syphilis	Bosnia A	0.0010 0.0022	0.0016
	Bosnia B	0.0017, 0.0007	0.0012

* Adapted from Nell [1]

[a] IC_{50} = penicillin concentration at which 50% of treponemes were immobilized

It will be noted from Table LXIV that the effective concentration for all the strains tested is about the same, to judge from the mean figures for several assays It is doubtful if any of the differences can be regarded as significant and certainly there is no evidence suggesting that any of these strains have an abnormally high resistance to penicillin

more organisms in 200 fields to less than 10 treponemes in 200 fields, within 24 hours after the initiation of treatment The total dose was given in 3 equal amounts 2 hours apart. Turner, Cumberland & Li[2] also reported that half this dose (0 125 mg) will reduce the count to 10 treponemes or less in approximately one half of the animals. It was further found that a total dose of 0.025 mg/kg body-weight rarely reduced the count to this level when the Nichols strain was the test organism.

In the comparative tests with various strains, carried out in collaboration with our associate, Dr Katherine Schaeffer, dosages of 0 25 mg and 0 05 mg of penicillin G were given. Difficulty was experienced with some strains in obtaining lesions with sufficient treponemes for the *in vivo* tests, so that many animals which were inoculated for this purpose were not used. The results shown in Table LXIII represent only completed tests.

TABLE LXIII COMPARATIVE SENSITIVITY OF NEWLY ISOLATED STRAINS OF TREPONEMES TO PENICILLIN G AS DETERMINED BY A SHORT "IN VIVO" METHOD

Group	Strain	Results of indicated dose[a]	
		0 25 mg/kg body weight	0 05 mg/kg body-weight
Syphilis	Nichols	1/12	10/11
,,	Chicago	1/3	2/3
,,	Baghdad A	0/3	2/4
,,	Baghdad B	0/3	3/3
,,	Mexico A	0/3	1/2
Yaws	Haiti A	0/2	
,,	Haiti B	1/4	2/3
,,	Indonesia B	0/2	0/2
,,	Samoa D	0/3	1/3
,,	Samoa F	1/1	
Bejel	Syria A	1/3	2/3
,,	Syria B	0/2	0/1
,,	Iraq B	0/1	1/1
Endemic syphilis	Bosnia A	0/3	2/3
,,	Bosnia B	0/2	0/1

[a] Numerator = number of rabbits in which treponeme count remained higher than 10 in 200 fields, denominator = number of rabbits in which tests were made

Part III

RECAPITULATION AND DISCUSSION

REFERENCES

1 Nell, E. E. (1954) Comparative sensitivity of treponemes of syphilis, yaws and bejel to penicillin in vitro, with observations on factors affecting its treponemicidal action, *Amer J Syph*, 38, 92

2 Turner, T B, Cumberland, M. C. & Li, H.-Y. (1947) Comparative effectiveness of penicillins G, F, K, and X in experimental syphilis as determined by a short in vivo method, *Amer J. Syph*, 31, 476

Part III

RECAPITULATION AND DISCUSSION

Chapter 10

RECAPITULATION AND DISCUSSION

Most of the studies included in this monograph represent the investigations conducted in this laboratory during the past decade, but a few are of an earlier date. One wishes that much more had been accomplished, yet on the whole it can fairly be stated that our knowledge of the treponematoses is now greater than it was, and that this knowledge inevitably will help in the very practical problem of the control of these diseases, leading it is hoped to fewer cases of infection and less suffering among the afflicted

In this chapter we shall summarize the principal points brought out during the course of these studies, and in so far as it is possible we shall attempt to fit them into some reasonable pattern in terms of the fundamental biology of the treponematoses.

I. BIOLOGY OF TREPONEMAL INFECTIONS

Sources of Strains Studied

As was seen in Chapter 1 altogether 76 strains of treponemes have been isolated, 70 of these from human beings and 6 strains of cuniculi treponemes from rabbits Among the strains isolated from human beings were 39 from patients with a clinical diagnosis of syphilis, 20 from patients with yaws, 3 from bejel patients, and 8 from patients with one of the syndromes classified as endemic treponematosis While there were some failures in attempts at isolation, these failures occurred either early in our experience when less information about the factors that affect the results of isolation was available than at present, or under circumstances where sources of strains were plentiful, and one could therefore be less meticulous and use fewer animals than in situations where sources of treponemes were less abundant.

Viewing the experience as a whole we believe that the hamster is the most satisfactory laboratory animal in which to isolate treponemal strains All the human strains studied, with the exception of pinta treponemes, either induced lesions in the hamster at the site of inoculation, or else involved the regional lymph nodes, from which treponemes could easily be

which would permit better quantitation of experimental results It has often been possible to pursue these two objectives at the same time.

Following testicular or intracutaneous inoculation syphilis treponemes induce an indurated type of initial lesion Other species of treponemes induce a less indurated type of lesion; these differences are described more fully in Chapter 7.

The clinical evolution of experimental syphilis in rabbits has been correlated with the concomitant histopathological changes. Prominent among these is the initial production of a mucoid material identified as hyaluronic acid A second stage in the evolution of the syphilitic lesion is characterized by the influx of mononuclear cells probably as a manifestation of the immune response on the part of the host In a third stage polymorphonuclear leukocytic infiltration becomes prominent, presumably as a reaction to injury, to necrosis, and sometimes to secondary infection in the lesion Comparative features of the histopathology induced by different species of treponemes are discussed in Chapter 7.

Among the developments in respect of better quantitative methods were, first, the utilization of a " pattern " method of multiple intracutaneous inoculation, especially in immunity experiments. This method, by simplifying the reading of results, has the virtue of being much more precise from the standpoint of uniformity and reproducibility than the method of inoculation into the testis, eye, or bloodstream Secondly, studies were made on the rate of multiplication of treponemes in the hours and days following inoculation of rabbits It became clear from these studies that after a time lag of 24 to 48 hours, treponemes multiply in a logarithmic pattern, until interrupted by the immune processes of the host, or unless some other extraneous factor, such as the administration of antibiotics or an unfavorable environmental temperature, intervenes It is in the nature of these methods that the more that is known about the optimum conditions for the multiplication of treponemes the more precise can be the quantitation when any single factor is examined

From these studies it has become clear that the incubation period can be regarded as an index of the number of viable treponemes inoculated, and this constitutes an indirect method of some value in estimating the number of viable treponemes in a given inoculum

On the basis of extensive observations in this laboratory, it has been shown that there is a direct straight-line relationship between the number of treponemes (Nichols strain of *T. pallidum*) inoculated into rabbits and the incubation period of the resulting lesions With an intradermal inoculum of 500 treponemes per site the incubation period is approximately 17 days; for each tenfold increase or decrease in the number of treponemes the incubation period is shortened or lengthened, respectively, by about 4 5 days. A generation time of 30-33 hours for *T pallidum* can be computed from these data.

recovered. Transfers of hamster material to rabbits then usually resulted in the establishment of the strain in the latter species.

Direct transfer of material from man to the rabbit was likewise successful in a high proportion of instances. On the whole inoculation of the material into the body of the testis yielded the best results, but when secondary contaminating organisms were present, purulent lesions often developed in the testis, thus reducing the chances of successful isolation Injection of material into the skin of the back or by scarification into the skin of the scrotum usually circumvented difficulties due to secondary infection, but the proportion of positive results tended to be lower than when intratesticular inoculation was used In attempting to isolate strains in rabbits it is recommended that all three routes be employed.

In most of the inoculations of hamsters in this laboratory, material has been injected intracutaneously into the groin region. There is no proof that this area is especially favorable, and yet our impression is that it is somewhat more satisfactory than the skin of the back, for example. In the first place, the fur of the animals is less abundant in the former area, so that a developing lesion can be better visualized. Moreover, the lymph drainage from this area is into the inguinal lymph nodes, which are readily accessible at operation Finally, the animal can conveniently be grasped by one hand and inoculated with the other.

At times inoculations have been made into the lips of the hamster, but we do not recommend this because the resulting lesions often interfere with the animal's eating, and it increases the danger of laboratory infection in the event of laboratory personnel's being inadvertently bitten by the animal.

Our experience in the attempted isolation of pinta treponemes has been disappointing but perhaps not altogether hopeless for the future. Transfers have been made from 3 patients into a total of 30 hamsters and 2 rabbits. As pointed out in Chapter 1, darkfield examination revealed the presence of treponemes in the regional lymph nodes of 3 hamsters, but subsequent passages into hamsters were negative. Perhaps other attempts should be made using not only hamsters and rabbits but other laboratory animals as well.

Pertinent information on patients from whom the newly isolated strains of treponemes were obtained is given in Appendix 1.

The Experimental Disease in Laboratory Animals

Much, of course, was already known about the characteristics of experimental treponemal infections in laboratory animals, and our own efforts, described in Chapter 2, have been directed largely to the exploration of poorly understood aspects.

In studying the experimental disease in rabbits attention has been directed, first, to investigation of certain fundamental biological processes relative to treponemal infections, and, second, to the development of techniques

Limited studies on the course of experimental treponemal infections in guinea-pigs and mice are also presented

From a more fundamental standpoint, our studies have emphasized the critical importance of hyaluronic-acid-like substances in the pathogenesis of treponemal infections. While this mucoid material has repeatedly been described in the pathological process of syphilitic infections, we were perhaps the first to suggest, on the basis of some experimental and circumstantial evidence, that hyaluronic acid is probably produced by the treponeme itself and is in the nature of capsular material; that there may be a direct relationship between the abundance in which this material is produced and the virulence of a particular strain of treponeme; and that this attribute may be one of the principal differences among treponemal strains of various origins. It is further suggested that the conversion of hyaluronic acid to a sulfated form, which partakes of the characteristics of chondroitin, may be related to the damage and scarring of tissues following infections, and this supposition leads in turn to a hypothesis that the strains of treponemes which produce the most hyaluronic acid can ultimately cause the most damage to the animal or human body Finally, from these studies has come a conviction, as yet not substantiated by adequate experimental evidence, that the conversion of mucopolysaccharide to a sulfated form, as postulated for treponemal infections, will provide a model for certain other infectious processes, notably streptococcal infections, in which the same sequence of events may occur

Factors Affecting the Evolution of Experimental Treponematosis

The study of factors (other than the parasite or the host *per se*) which influence the course of treponemal infections in laboratory animals was chiefly concerned with environmental temperature, certain hormonal effects, antibiotics and related substances, and the early operation of the immune mechanism. These investigations are discussed in Chapter 3.

Environmental temperature

Our studies on the effects of temperature in experimental treponemal infections were initiated largely with the limited objective of determining at what temperature the treponeme seemed to thrive the best *in vivo* As usually happens, however, the results of these studies had implications beyond the original objective.

To summarize a whole series of experiments, it appears that the environmental temperature favorable for treponemes is approximately 35°C. Even only slightly higher temperatures are unfavorable and when the

Direct treponeme counts made on rabbits' testes following inoculation with a known number of organisms reveals that after the first 24 hours there is a regular logarithmic increase in treponemes; computations based on these data likewise indicate a division time of 30-33 hours for the Nichols strain of *T. pallidum*.

Assuming that each treponeme divides into two, a single treponeme *multiplying logarithmically will yield about 100 000 000 organisms, the* number believed necessary to produce a macroscopically recognizable lesion in approximately 32 days These methods were applied in detail mainly to syphilis treponemes, but in broad outline the findings are probably valid for other strains of pathogenic treponemes.

The time of development of generalized lesions appears to depend on two factors· one of these is the time at which the treponemes migrate from the local focus, a process which probably begins within the first few hours or days of infection and continues until retarded by the development of the immune mechanisms, the other factor is the time required for the multiplication of the few treponemes at the remote focus.

From a technical standpoint our exploitation of the hamster as an experimental animal has given rise to many practical advantages in the isolation and maintenance of strains of treponemes. In addition, the use of the hamster has revealed strain differences among treponemes which, while as yet not fully understood, must be regarded as a consequence of underlying biological differences Three types of reaction in the hamster are recognized an Sh type characterized by few local lesions, but involvement of the regional lymph nodes; an Mh type in which both local skin lesions and involvement of lymph nodes are observed, and a Yh type in which local lesions are common but involvement of the regional lymph nodes rarely occurs

Limited studies on the monkey as an experimental animal for the treponematoses have been made. In the monkey syphilitic lesions at the site of inoculation tend to remain small; generalized lesions seldom occur and are not prominent *T. cuniculi* was shown to be pathogenic for the monkey, but the lesions produced, and indeed the whole disease process, were insignificant. The results suggest, however, that *T. cuniculi* may likewise be pathogenic for human beings, the potentiality of this organism as an immunizing agent against syphilis, yaws and related syndromes has not been fully explored.

For the study of most problems the monkey offers no advantages and indeed serious disadvantages in comparison with the smaller and less expensive rabbit and hamster However, there are undoubtedly problems which could be studied more definitively in monkeys than in these other laboratory animals; such studies should ideally be conducted where monkeys are plentiful and where they can be maintained under conditions of good general health and nutrition.

experience in this field, presented in detail in Chapter 3, has been limited largely to a study of the effect of certain of the steroid hormones, notably cortisone and related products

The general action of cortisone in animals and human disease conditions is now well known. Important among its effects are that it suppresses inflammatory tissue reactions of all kinds, including those due to hypersensitivity, in large doses it tends to suppress antibody production. Through one or both of the foregoing mechanisms, it tends to favor the excessive growth of many bacteria in the animal or human body.

These effects were early noted in experimental syphilis. The administration of cortisone to animals infected with syphilis leads to a tremendous over-growth of treponemes in both initial and secondary foci Concomitantly there is a great increase in the amount of hyaluronic acid in these lesions, which lose their typical firm, often stony-hard characteristics, becoming soft, spongy and filled with mucoid material

As mentioned in the preceding section, there is good indirect, but unfortunately not direct, evidence that this mucoid material is produced by the treponemes themselves. A hypothesis put forward from this laboratory, which has not yet been substantiated, postulates that under ordinary conditions this hyaluronic acid is converted rather rapidly to a sulfated compound similar in its properties to chondroitin sulfate, under the influence of cortisone, according to the hypothesis, the sulfating of hyaluronic acid is inhibited, thus giving rise to the characteristic changes observed in these lesions

It has been suggested, too, that an increase in the amount of hyaluronic acid may favor the growth of treponemes At the same time there is perhaps a less favorable situation from the standpoint of the defenses of the body, in that phagocytosis may be inhibited by the mucoid material, and even the penetration of antibody may be inhibited It is well known that in large doses cortisone suppresses antibody production, but the changes noted in treponemal lesions occur so rapidly after the initiation of steroid therapy that this phenomenon does not appear to play a major role in the appearance of such changes

Withdrawal of cortisone in experimentally infected animals often leads to the so-called rebound phenomenon in which there is a rapid and tremendous increase in the size of lesions, often with development of extensive generalized lesions. This phenomenon can probably be explained by the presence in the lesions of enormous numbers of treponemes, which are then *freed from the suppressing effect of cortisone.*

The demonstrated action of cortisone in experimental treponemal infections has been put to practical use in all procedures in which large numbers of treponemes are desired, such as the preparation of treponemal antigens Cortisone has also been used in attempts to enhance the virulence of newly isolated strains of treponemes, but with less successful results

temperature rises to near 40°C progressive destruction of the treponemes occurs. Likewise as the temperature falls inhibition is noted, although the lower limits of multiplication of treponemes have not been so well defined as the upper The evidence indicates that pathogenic treponemes probably multiply slowly if at all at temperatures below 30°C.

Most of these studies were made with the Nichols strain of *T. pallidum*, but the evidence suggests that other species and strains of treponemes are influenced in much the same manner as the Nichols strain; and while most of the studies on the effect of temperature were made on the experimental disease in rabbits, it appears that the same factors are also operative in hamsters.

From these investigations has come the realization that the localization of treponemal lesions is influenced perhaps in a major way by the local temperature of the animal or human host The internal body temperature of the rabbit, for example, is normally about 39°C—a temperature which is higher, of course, than that of man—and lesions of internal organs of the rabbit are rarely if ever encountered. By contrast, however, certain areas of the rabbit's body, notably the skin, ears, testes and extremities, are significantly cooler than the internal body temperature and it is in these areas that treponemal lesions occur readily, either as a result of direct inoculation or by metastasis from focal lesions elsewhere.

One can only speculate concerning the effects of long-continued environmental temperature on the treponematoses in human beings It can be deduced from these studies on the experimental disease that consistently high environmental temperatures such as are met with in the tropics, which in turn account for slight but definitely higher skin temperatures, may have a slightly adverse effect on treponemes infecting individuals inhabiting the areas concerned, and this thesis can reasonably be extended to visualize a substantial modification of those strains of treponemes, after years and years of exposure, towards a less virulent variety. Carrying this idea a step further it may be postulated that treponemes of lowered virulence likewise provide a lesser antigenic stimulus to the host. This in turn may account for poor development of the immune response, with a tendency to chronicity and relapse over a period of many years.

Hormonal effects

Earlier studies in other laboratories have indicated that male rabbits tend to exhibit a more extensive disease picture of experimental syphilis than do female rabbits. Moreover, the administration of female sex hormones (estrogens) to male rabbits induces a milder type of disease than is seen in normal male controls. These studies were made before some of the modern techniques of quantitating treponemal infection were developed, so that the differences are often not too sharp or too definitive. Our own

The influence of prior cuniculi infection

As pointed out in Chapters 7 and 8, there is a substantial degree of cross-immunity between the treponematoses of man and the natural rabbit disease caused by *T. cuniculi*. Pre-existing cuniculi infection of laboratory rabbits therefore significantly modifies the response of these animals to inoculation with other species of treponemes. Indeed, at times it appears to account for the complete suppression of lesions in supposedly normal rabbits.

Pre-existing cuniculi infection has been particularly troublesome in the production of treponemal antigens for the treponemal agglutination test or the treponemal immobilization test, since naturally occurring cuniculi infection appears to be widely prevalent among domestic rabbits in the United States of America The trouble seems to arise from the accelerated production of specific antibody following the initiation of the experimental infection, the treponemal inoculum serves as a booster dose in rabbits which have had what amounts to a basic immunizing infection, through the previously naturally occurring cuniculi infection While such animals customarily show negative standard serological tests, they can now be identified by prior testing of their serum with the treponemal agglutination test.

Other factors influencing the course of experimental treponemal infection

Considered also in Chapter 3 are other factors which are known to influence the course of treponemal infection in the rabbit, including the breed, age and sex of the animal, intercurrent infection in the host animal, and the site of inoculation As for the influence of breed, age and sex of the rabbit, little additional work has been done in this laboratory. The influence of intercurrent infections appears to rest largely upon the extent to which the body temperature of the animal is raised, thus bringing into play the adverse effect of elevated temperature on the treponemes, as mentioned above

The influence of site of inoculation likewise seems to depend largely if not entirely on the suitability of local conditions at the site, the skin and testes being favorable sites, because their temperature—several degrees below the internal body temperature—is particularly suitable

Inoculation of treponemes directly into the blood-stream of rabbits leads to a highly selective localization of lesions Although with large inocula treponemes are presumably carried to all parts of the vascular bed, lesions occur principally in the distal portions of the extremities, and on skin surfaces of the trunk from which the fur has been removed. The temperature in all of these areas is known to be lower than the internal body temperature of the rabbit, and it is concluded that the localization of the treponemal lesions is determined largely by this factor.

Antibiotics

The effect of antibiotics on treponemal infections is considered principally in Chapters 6 and 9, in Chapter 3 we are concerned more with the incidental effects that arise from contacts with antibiotics other than those intentionally employed for therapeutic or prophylactic purposes.

The very complexity of our civilization makes it extremely difficult to keep traces of unwanted elements from reaching the experimental animal, or indeed man This has been true of the antibiotics, for time and again one or another of these drugs has inadvertently been introduced, either directly or indirectly, into the food of our experimental animals. For example, many prepared animal foods contain antibiotics, either as a result of deliberate addition, or because they contain meat and milk products obtained from animals to which antibiotics have been fed. Repeatedly, our experiments or routine TPI tests have been ruined by the inadvertent and, at the time, undiscovered introduction of antibiotics into our animals' food

It is interesting to speculate on the extent to which the treponematoses are now being affected by the widespread use of antibiotics primarily for non-treponemal conditions. Nor is it altogether fantastic to postulate that even before antibiotics were discovered some of the geographical and epidemiological peculiarities of treponemal disease may have resulted from antagonistic bacterial or fungal flora in certain areas of the world.

Sensitization of treponemes

While the general picture of immunity in treponemal infections is presented in Chapter 5, one effect of immunity on the genesis of the infection is considered in Chapter 3. This occurs principally when small amounts of specific antibody, perhaps produced in or in proximity to the local treponemal lesion, become intimately associated with treponemes. Such sensitized treponemes, which constitute the earliest detectable evidence of an immune response, have a decreased vitality

When such treponemes are tested *in vitro* the mere addition of complement will induce immobilization of the treponemes To what extent a similar phenomenon takes place *in vivo* is difficult to determine, although it may be the basis for the earlier observations that when treponemes from long-standing infections are transferred to a new host the incubation period is commonly longer than that produced by approximately the same number of treponemes obtained from very early lesions.

This same phenomenon may account in part for the difficulty encountered at times in successfully transferring treponemes from one animal species to another; or in transferring the organisms to laboratory animals from human beings with long-standing infections. *In vivo* sensitization of treponemes can likewise be a problem in the preparation of antigens for the treponemal agglutination test or for the immobilization test.

of glycerol in a concentration of approximately 15% to the suspending medium appears to prevent this damage, and survival results are considerably superior to those obtained with aqueous suspension This same phenomenon is observed with many other species of micro-organisms.

Damage to treponemes during the storage period is however largely a function of temperature When frozen in 15% glycerol, pathogenic treponemes were non-infectious after one month's storage at −15°C, at −40°C there was good survival for one month but not for two months, while at −70°C an aliquot of this same material was not perceptibly diminished in virulence after 9 months.

The conclusion is reached on the basis of a review of the literature and the experiences in our own laboratory that no reliable method now exists for the cultivation *in vitro* of pathogenic treponemes. Studies on cultivation of the non-pathogenic Reiter spirochete are described and the various forms of the organism observed during successive stages of cultivation are illustrated

Immunity Phenomena in the Treponematoses

Because of the peculiar chronicity of syphilis, the long-continued precarious balance between host and parasite, the inability readily to demonstrate serum antibodies in infected humans or animals, and perhaps because of the enormous scientific authority of Neisser's observations, the treponematoses, particularly syphilis, were regarded for many years as being immunologically unique among infectious diseases The work of Chesney and his associates and, stemming in a straight line from Chesney's findings, the studies in this laboratory, have had the effect of bringing these diseases once again into an immunological pattern common to other infectious processes

[illegible] development of several serological tests which have come to play an important role in the clinical management of patients with one or another of the treponematoses, particularly syphilis

Evolution of the immune state

In Chapter 5, after outlining briefly some of the earlier work on the evolution of the resistant state in the treponematoses we have illustrated these phenomena by experiments reported from other laboratories as well as our own From these experiments, which deal almost entirely with experimental syphilis, certain basic facts emerge, the most noteworthy of which are the following

Characteristics of Treponemes "In Vitro"

Since pathogenic treponemes cannot be cultivated on artificial media, all *in vitro* studies of this organism must be made with material containing relatively large amounts of host tissue, thus complicating antigenic studies and chemical analyses. The methods and problems of *in vitro* studies are examined in Chapter 4.

Large numbers of treponemes for experimental purposes can best be obtained from testicular lesions of rabbits following the use of cortisone. Methods of separating treponemes from host tissue are described.

Recent studies with the electron microscope (carried out in laboratories other than our own) confirm earlier observations concerning the presence of an axial filament, and give validity to Noguchi's classical description: "The essential structure of a treponema is a spring-like axial filament and a layer of contractile protoplasm enclosed in a delicate periplast"

It is clear that treponemes may exhibit capsular material, and this is probably made up largely of hyaluronic acid. Characteristic spiral motility is snake-like in viscid media, and rotatory in more fluid media. Mention is made of miscellaneous observations on the staining of treponemes, on centrifugation, and on the refractive index of pathogenic treponemes.

A medium which permitted the survival of pathogenic treponemes for periods up to two weeks has been developed in this laboratory and modified here and elsewhere This development has paved the way for the demonstration of treponemal immobilizing antibody. Subsequent study of this "survival medium" has shown that, while the media must be anaerobic in nature, the maintenance of anaerobiosis is not sufficient, it appears that compounds containing sulfhydryl groups are essential, possibly because these compounds participate directly in the metabolism of the treponemes.

Studies on the survival of treponemes *in vitro* were undertaken in the hope that they would provide a rational approach to the cultivation of the organism on artificial media It may be, however, that optimum conditions for survival are altogether different from those required for multiplication For example, it seems probable that factors, such as decrease in temperature, which slow the metabolic rate of treponemes, tend to prolong survival, and yet these same factors may well be unfavorable for multiplication

To the earlier studies on long-term survival of treponemes in the frozen state have been added new observations of greatly enhanced survival. When frozen at approximately $-70°C$ both syphilis and yaws treponemes have been found to be virulent for rabbits after storage for over 9 years. However, when frozen in aqueous suspension the treponemes undergo a considerable loss in viability due probably to damage at the time of either freezing or thawing rather than during the storage period. The addition

can be made for strains of treponemes belonging to the yaws, bejel, and endemic treponematosis groups.

Humoral expressions of immunity

From this laboratory has come the demonstration that human beings and animals infected with treponemes develop antibodies that are specific for this group of organisms. It is interesting to recall that Wassermann, in his original investigations on syphilis serology, was looking for a rich source of treponemes to serve as a specific antigen, and extracted fetal liver from congenital syphilitics for that purpose. The presence of the same or a cross-reacting alcohol-soluble substance in normal tissue led to the substitution of this more convenient but perhaps less specific material as a diagnostic antigen Concentrated attention on the tissue antigen over a period of years has progressed to the point of chemical identification of a serologically active phospholipid fraction in normal tissue. In the past few years attention has been re-directed to the study of the treponeme itself as a potential antigen, thus in a sense completing the circle initiated by Bordet and Wassermann and their associates.

The earliest work in this laboratory on the general subject of humoral immunity resulted in the demonstration of the suppressive effect of serum from syphilitic animals and human beings on the development of lesions, when serum and treponemes were mixed *in vitro* and inoculated into test animals The development of methods for maintaining motile treponemes *in vitro* over a period of days made it possible to observe the effects of normal and immune sera on these organisms without resorting to the more cumbersome method of animal inoculation From this evolved the treponemal immobilization test which has been widely used both in clinical medicine and in the study of certain fundamental aspects of the treponematoses.

Considered from a basic biological standpoint, the TPI test clearly measures an antibody to pathogenic treponemes which differs from the so-called Wassermann antibody In the vast majority of patients infected with one of the treponematoses and in many experimentally infected animals the two antibodies exist simultaneously, although often in differing quantities

Existing evidence indicates that immobilizing antibody is a highly specific index of past or present treponemal infection; it has also been invoked by the injection of killed treponemes. There is evidence, gained mostly from study of the experimental disease, that treponemal immobilizing antibody plays a significant role in immunity to treponemal infections. However, enough discrepancies are noted between the presence of immunity and the presence of immobilizing antibody to indicate that this antibody is perhaps not the sole factor in the immune process.

At the clinical level the TPI test has proved to be a noteworthy aid in the management of treponemal infections, and there are some indications that

Immunity in syphilis develops comparatively slowly over a period which is best measured in weeks rather than in days, as in most of the acute infections This period is determined in part by the extent of the infectious process since this determines the degree of antigenic stimulus. Experiments were reported from this laboratory in which a minimal infection was maintained in the rabbit for periods up to 20 weeks, without the induction of significant degrees of resistance. In other words, the mere presence of infection is not sufficient in itself to stimulate the development of immunity.

The persistence of the immune state is dependent upon a number of factors. Once immunity has developed to its highest point—ordinarily within 3 months after initial infection—a high level of resistance is usually maintained as long as the infection, even though latent, is present.

The persistence of immunity, however, after elimination of infection by therapy seems to be a function of time plus the degree of immunity attained at the time of treatment For example, it was shown that a group of rabbits treated 2 months after infection and challenged shortly after the termination of treatment had a significantly higher degree of immunity than another group of animals similarly inoculated and treated but challenged one year after treatment. Animals in which immunity is fully developed at the time of therapy, however, commonly show no evidence of diminished resistance after one year.

The whole question of latency in treponemal infections remains rather an enigma. Rabbits with untreated syphilis may show a high degree of immunity to challenge inoculation, as indicated by the absence of lesions, yet at the same time they may be harboring virulent treponemes in their lymph nodes immediately prior to challenge inoculation. The same phenomenon can be observed in treated immune syphilitic rabbits, in which, despite the failure of lesions to develop on challenge inoculation, latent infection may be established It is difficult to envisage the biological mechanism that is the basis of this phenomenon, whereby the defenses of the host are capable of restraining the invading parasite to the point where it can do no harm, but at the same time are incapable of destroying the parasite altogether. The study of treponemes from animals with latent syphilitic infection has failed to reveal biological differences in these organisms that might account for their seeming resistance to the immune defenses of the host

A new observation stemming from the experiments referred to above is that reinfection in rabbits with a high degree of immunity seems to occur largely without reference to the size of the challenging inoculum, within the limits of the experimental techniques employed. Again, it is difficult to explain the biological basis of this observation

While most of the investigations on the evolution of the immune process in treponemal infections have been carried out with the Nichols strain of *T. pallidum*, what evidence there is indicates that other strains of syphilis treponemes behave in essentially the same manner. The same statement

and upon centrifugation are cleared from the supernatant fluid; but they remain in suspension in mixtures containing normal serum.

Current studies in this laboratory indicate that the red blood cell adherence is essentially a manifestation of the type of adhesion reaction, described by Rieckenberg, between trypanosomes and specific antibody in the presence of complement. Adhesion of this character has been demonstrated between treponemes and erythrocyte ghosts and blood platelets from man, the rabbit and the guinea-pig; collodion particles, and the bacteria *Escherichia coli*, *Alkaligenes faecalis*, *Streptococcus pyogenes*, *S. lactis*, and *Spirillum rubrum* in the presence of complement and syphilitic immune sera. The difficulties inherent in such complicated systems, however, have not been fully examined, and the uses and limitation of this phenomenon as a practical test remain to be explored

Published reports concerning the utilization of serologically active chemical fractions of pathogenic treponemes are not yet detailed enough to determine the potential value of this approach.

Finally, it should be noted that newer methods of extraction of treponemes have permitted the development of better antigens for use in skin-test procedures While studies of this nature are in preliminary stages they already confirm and strengthen older observations to the effect that among persons with syphilis a large proportion of those who have late syphilis show a positive skin reaction of the tuberculin type, while most of those with early syphilis, as well as normal persons, do not

Response of Treponemes to Drugs

In studying the comparative effectiveness of various drugs, particularly the antibiotics, on treponemal organisms, and the comparative response of various strains of treponemes to the same antibiotic (Chapter 9), it was necessary first to develop satisfactory methods of assay, since the methods previously used were expensive and time-consuming

Both *in vivo* and *in vitro* methods have been developed in this laboratory; while these procedures, which are described in Chapter 6, are perhaps accurate only within a two- to threefold range they seem to be as good as any other heretofore used, and have the virtue of being comparatively simple and inexpensive.

In the *in vivo* method developed in this laboratory treponemal lesions are induced by intracutaneous inoculation of the strain of organisms to be tested When these lesions have reached their maximum development, treponemal counts by darkfield examination of material taken directly from the lesions are made before and after the administration of graded doses of the antibiotic to be tested Critical readings are customarily made 24, 48 and 72 hours after administration of the first dose of the drug

it may have even wider application in the study of other chronic disease processes

Because Wassermann antibody occurs in some human beings in whom all the clinical and epidemiological evidence indicates the absence of past or present treponemal infection, the TPI test has been a valuable means for identifying these so-called biologic false positive Wassermann reactors There are suggestions that the presence of detectable amounts of Wassermann antibody in persons who have never been infected with one of the treponemal organisms may be indicative of some other underlying disease which in time will become clinically overt At the time of writing, however, we are only on the threshold of knowledge in this field

Because of the complicated technical features of the TPI test, intensive search has been made in this laboratory for other methods of detecting specific antibody to pathogenic treponemes. Among the approaches made have been (*a*) attempts to simplify the TPI test, without however signal results, (*b*) study of the phenomenon of treponemal agglutination as a specific antigen-antibody reaction, and (*c*) study of adhesion phenomena In addition, attempts in another laboratory to obtain a serologically active chemical fraction from pathogenic treponemes appears at the time of writing to have met with some success

With the use of cortisone to enhance the *in vivo* growth of treponemes, and with improved methods of extraction and separation of treponemes from tissue elements, it has been possible to prepare highly concentrated suspensions of treponemes suitable for agglutination studies

These treponemal suspensions, when killed by heat, agglutinate both with Wassermann antibody and with another and seemingly specific antibody probably identical with treponemal immobilizing antibody. In the agglutination test developed in this laboratory Wassermann antibody is first removed from the serum by absorption with cardiolipin antigen or crude powdered beef-heart, thus leaving the specific antibody to react in the agglutination test.

Difficulties have been experienced in the preparation of agglutinating antigens, since some batches are quite satisfactory while others are not. The factors influencing these results have not been fully identified While, therefore, much valuable information has been acquired through the use of the treponemal agglutination test, unfortunately at the present time it is not sufficiently standardized as a test procedure to render it useful as a routine diagnostic method However, the simplicity of the test procedure commends it for practical use, provided that the more fundamental difficulties are overcome.

The immune adherence phenomenon described from this laboratory,
[illegible] ddition of treponemes
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to what extent exposure to these minute traces of antibiotics over long periods of time may modify the picture of treponemal disease can be only a subject for conjecture. It is possible even that fundamental changes might be induced in a strain of treponemes by this means; but factual data on these points will be extremely difficult if not impossible to acquire.

Attempts to induce increased resistance to penicillin in one or another species of treponemes under experimental conditions have given no indication that such a phenomenon does or can occur. It is known that some micro-organisms, particularly the staphylococci, readily develop resistant forms, probably as selective mutational phenomena, while other organisms, as for example the hemolytic streptococcus, seem to develop little or no increased resistance to penicillin under either natural or experimental conditions. It will be a fortunate circumstance if in this respect treponemes behave like the latter groups of organisms

Studies on the mode of action of penicillin on pathogenic treponemes have failed to reveal the basic nature of this phenomenon, although some useful knowledge has been acquired. From *in vitro* studies it has been found that regardless of the dose of penicillin there is a time-lag of a minimum of about 4 hours before any treponemicidal effect can be noted After this, however, the rate of action of penicillin is a function of the concentration of the antibiotic and of the temperature.

In the case of staphylococci it is known that penicillin acts principally on organisms that are actively metabolizing, rather than on those which are more or less in a resting stage. Good data on this point in respect of treponemes are not available. Since all suspensions of pathogenic treponemal organisms are obtained from animal tissues it is assumed that any one suspension will contain treponemes in various stages of growth and reproduction All treponemes, however, appear to be susceptible to penicillin when exposed to sufficiently large doses, with borderline amounts of penicillin, there is a substantial differential in time between the first and last treponemes to be immobilized, but the factor or factors determining this differential susceptibility are not understood

From both *in vitro* and *in vivo* experiments it appears that penicillin exerts its lethal effect on treponemes without bringing about lysis True enough, in treponemal lesions of man or of experimental animals a marked reduction in the number of treponemes is commonly observed after appropriate penicillin therapy. However, in infected animals treated with cortisone in which there are excessive amounts of mucoid material in the lesions, penicillin renders the treponemes immobile and presumably dead, without effecting a substantial decrease in their number This suggests that the essential action of penicillin does not invoke the phenomenon of lysis; it is postulated that dead treponemes are normally cleared from lesions through phagocytosis, which is inhibited in the cortisone-treated animal by the presence of large amounts of mucoid material and decreased numbers of phagocytes.

The *in vitro* method developed was patterned after that used for certain other bacteria, although adaptation of the method to the study of treponemes required the utilization of specialized and on the whole rather complicated procedures The method consists essentially of extractions of pathogenic treponemes from rabbits' testes, separation of the organisms from tissue debris in so far as is practicable, and maintenance of the treponemes in a medium that will permit retention of motility for at least 18-24 hours. The particular antibiotic to be assayed is then introduced into this system. A useful end-point is that concentration of drug which immobilizes (and presumably kills) 50% of the treponemes within a given period of time, commonly 18 hours. This method has been used principally in determining the comparative susceptibility of various strains of treponemes to graded doses of penicillin, the results are presented in Chapter 9.

With the use of the *in vivo* method referred to above various antibiotics and certain other therapeutic agents were tested against the Nichols strain of *T. pallidum*. Of the penicillin fractions assayed, penicillin G was found to be significantly more active than fractions F and X, and much more effective than fraction K. It is of interest to note that the results obtained by this short *in vivo* method of assay were similar from a comparative standpoint to those obtained by much more time-consuming and expensive methods of *in vivo* assay.

Assays of some of the newer antibiotics by this short *in vivo* method showed that while none was as effective as penicillin, a number did have significant therapeutic potentialities against the Nichols strain of *T. pallidum*, and, in view of the results of *in vitro* studies presented in Chapter 9, presumably against other species and strains of treponemes as well.

Among the newer antibiotics tested Magnamycin was the most active, while Aureomycin, Terramycin and erythromycin were therapeutically active in doses approximately 100 times greater than that of penicillin on a mg/kg body-weight basis Chloromycetin and streptomycin on the other hand were only slightly active in much larger amounts. Magnamycin and penicillin when used together appeared to exhibit an additive effect.

In view of the wide range of antibiotics that have some bactericidal or at least some inhibitory effect on treponemal infections, it is probable that these diseases in man are continuously being subjected to minor therapeutic effects. What role this may play in their control or modification is difficult to assess. In areas where penicillin is widely available, as is now the case in many countries, its widespread use through lay as well as medical channels may well be highly influential in modifying the epidemiological and even the clinical picture of these diseases.

In addition to the use of antibiotics in the treatment of human disease it must not be overlooked that antibiotics are now widely used in the dairy livestock and poultry industries, so that milk and meat products, in certain geographical areas at least, often contain traces of these substances Again,

wa Strains of cuniculi treponemes isolated from the naturally occurring diseases in rabbits seem to produce a disease picture in both hamsters and rabbits which differs somewhat from the foregoing ones, although studies on this group of strains are not definitive. These strains have been tentatively designated as the C type

In an effort to apply these particular indices in a quantitative manner, each of the 17 newly isolated strains of treponemes, together with several older laboratory strains, has been analysed according to the type of reaction which they invoke in rabbits and hamsters A summary of these findings will be found in Table XLVII, page 199

It would doubtless be gratifying if each of these strains fitted neatly into the classification which we have devised Such is not the case, however, for although by and large the strains do follow a pattern roughly corresponding to the clinical and epidemiological syndromes from which they were isolated, the classification of strains by rabbit reactions is not identical with the classification obtained by hamster reactions Thus, only 3 of 4 strains isolated from typical cases of venereally acquired syphilis conform to the S type in both rabbits and hamsters, and only 3 of 7 strains from typical yaws cases have shown the extreme yaws type of reaction in both rabbits and hamsters. It can be stated that many other syphilis strains isolated from patients living in North America or Jamaica (see Tables IA and IB, Chapter I, pages 21, 22), but not included in this analysis, likewise conform to the Sr type as observed in rabbits, but most of these latter strains were never observed in hamsters

We have, therefore, ill-defined but none the less real differences in certain biological characteristics of these treponemal strains. Furthermore, such characteristics seem to be reasonably stable, although rapid passage of a strain of the Yr type using large inocula often leads to a progressive increase in the proportion of animals showing the Mr or Sr type of lesion The fundamental basis for these observed differences is not clear, nor do we know what circumstances have led to their development There is some evidence suggesting that the key to the differences in behavior in rabbits is the ability of the strain of treponeme to produce hyaluronic acid, and in general more hyaluronic acid is present in lesions of the Sr type We have no corresponding hypothesis to offer as a possible explanation for the difference in behavior of these strains in the hamster.

The study of the histopathology of lesions induced by each strain of treponeme adds little to the information derived from observation of the gross lesions in rabbits and hamsters. There are no qualitative differences in the tissue response to the different strains, which are indistinguishable on the basis of histology alone. Nevertheless, real quantitative differences do occur, and these also seem to be largely a reflection of the amounts of hyaluronic acid produced by the treponemes. In consequence the relative amounts of metachromatic staining material, and the relative amounts of

II. COMPARATIVE STUDY OF TREPONEMAL STRAINS

Comparative Characteristics of the Experimental Disease Invoked by Various Strains of Treponemes

It has been observed in this and other laboratories over many years and by a succession of investigators that there are qualitative differences in the disease picture invoked in rabbits by different strains of treponemes These differences are examined in Chapter 7.

At one end of the scale there are strains which when inoculated either by the intratesticular or by the intracutaneous route quite uniformly invoke extensive lesions which are characterized by the presence of firm indurated tissue, having at times the consistency of cartilage. This type of reaction in rabbits has been referred to as the syphilis or Sr type, since it was first observed with strains derived from typical cases of venereally acquired syphilis. At the other end of the scale are strains of treponemes which commonly invoke only minimal lesions when inoculated intratesticularly or intracutaneously into rabbits; these lesions rarely contain the indurated tissue referred to above. Moreover, on testicular inoculation a peculiar granular involvement of the surface of the testis is often observed The strains that belong at this end of the scale are more difficult to maintain in rabbits, and on the whole can be clearly distinguished by all observers from the syphilis or Sr type strains This type of reaction has been designated as the yaws or Yr type, since it is most often observed in rabbits inoculated with strains of treponemes derived from typical cases of yaws. Strains of treponemes which appear to be intermediate between the Sr and Yr types have been designated Mr types.

Clear differences have also been noted in the reaction produced by various strains in hamsters. The Nichols syphilis strain and many other strains isolated from typical cases of venereally acquired syphilis commonly induce no lesion at the site of intracutaneous inoculation, although the regional lymph nodes show large numbers of treponemes. This disease picture in hamsters has been designated the Sh type. By contrast, strains of treponemes isolated from patients in Western Samoa with typical signs of yaws invoke in hamsters large, spreading lesions at the site of intracutaneous inoculations, with few or no treponemes demonstrable in the regional lymph nodes This disease picture in hamsters has been designated, perhaps rather arbitrarily, as the Yh type. A third type of reaction, which partakes of some of the features of each of the other two types, is characterized both by large, spreading lesions at the site of intracutaneous inoculation and by the presence of large numbers of treponemes in the lymph nodes. This disease pattern has been designated the Mh type, and has been observed with a number of strains isolated from patients with yaws, and with all the strains isolated from such syndromes as endemic syphilis, bejel and dichuch-

those used in the study of many other organisms— crude, primarily because pathogenic treponemes can be obtained only from mammalian tissue, and even the most highly "purified" concentrates of treponemes still contain proportionately larger amounts of host cellular material.

Despite these limitations, useful information has been obtained, first, through cross-immunity studies and, second, through the utilization of *in vitro* antigen-antibody reactions.

Relationship as revealed by cross-immunity studies. Cross-immunity studies have been made principally by testing newly isolated strains of treponemes against a single "standard" reference strain—the Nichols strain of syphilis treponemes—on the basic assumption that strains which show some degree of cross-immunity with the Nichols strain will likewise show some degree of cross-immunity with each other. Even with such a simplified system, cross-immunity tests carried out in rabbits are time-consuming and expensive, thus imposing limitations on the extent to which conclusive answers can be obtained.

Perhaps the most impressive result of these studies has been the clear demonstration that there is some degree of reciprocal immunity between all the pathogenic treponemes studied, even between strains isolated from the natural rabbit disease (non-venereal spirochetosis) and strains isolated from typical cases of syphilis in man. It can be stated unequivocally therefore that all the treponemal strains studied have some antigenic components in common.

The degree of cross-protection varies from strain to strain, despite the limitations of the test methods some notion of qualitative relationships can be obtained. For example, it is clear that the *cuniculi* strains have a lesser degree of reciprocal immunity with the Nichols than do the other strains of treponemes tested. It is not always certain, however, to what extent quantitative factors enter the picture, since in general the syphilis strains gave better immunity against the *cuniculi* strains than the *cuniculi* strains gave against the syphilis strains.

Altogether 23 strains isolated from patients with typical venereally acquired syphilis, most of them living in North America, have been tested against the Nichols strain in this laboratory. Of these, 18 showed a high order of cross-protection, in 2 data were equivocal or inadequate, while 3 strains, all from the United States, showed significantly lower degrees of cross-protection. The experience of other laboratories, while less extensive in this respect, indicates also that most syphilis strains show good cross-protection with each other. It would seem to be a fair summary statement that the vast majority of syphilis strains are sufficiently closely related antigenically to give good cross-immunity by the usual infection-challenge test in rabbits, strains do exist, however, which seem to be less closely related antigenically than the majority.

cellular infiltration present in lesions may also be a rough guide to the classification of the strains.

Observations have been made which are relative to the question of variation and mutation of strains upon continued passage in laboratory animals It is noted that at least one strain—the Nichols syphilis strain—has been shown through accidental laboratory infections to be still pathogenic for man 42 years after its original isolation in rabbits.

With rapid animal-passage most treponeme strains appear to assume enhanced virulence for the rabbit, to the extent that the incubation period of the initial lesion becomes shorter and the lesion becomes larger and more indurated. Strains of the yaws type tend to produce a higher proportion of lesions resembling the intermediate and syphilis types. Commonly there is no indication that these changes reflect a permanent alteration in the basic characteristics of the strain. Likewise, the maintenance of two typical syphilis strains in rabbits subjected to a high environmental temperature for 10 passages over a period of one year failed to induce recognizable changes in the strains when tested at the end of that period in animals subjected to a low environmental temperature.

However, in two instances changes believed to be in the nature of mutations have been observed, each involving the conversion of a yaws-type strain to one of a syphilis type The first observation was made by Chesney with strain Y9, which had been isolated in Haiti. This strain had been propagated in rabbits for three years through 9 passages, when transfers were made from the lymph nodes of rabbits with a long-standing infection, subsequently the lesions induced by two sub-strains approached in character those invoked by a syphilis strain which had also been isolated in Haiti.

The second observation of this nature involved our YD strain. Beginning with the 16th passage in rabbits this strain began to induce in some animals lesions resembling those of syphilis; the reaction in hamsters at this time was of the Mh type, which is often produced by yaws strains. A sub-line YD-post-1949 derived from the lymph nodes of an animal of the 26th passage, 27 months after its initial syphilis-like infection, invoked syphilis-type reactions in all subsequent transfers both in rabbits and in hamsters.

The suggestion is offered that long-standing infection of rabbits tends to select from populations of yaws treponemes those organisms having characteristics of syphilis treponemes.

Antigenic Relationship between Strains of Treponemes

Comparison of newly isolated strains of treponemes is continued in the material presented in Chapter 8, the approach here being directed to a study of the antigenic relationship existing between strains or groups of strains. Methods available for such investigations are crude when compared with

definitive, partly because we believed that some of the more important of these problems can be examined best in the monkey and the higher apes, and there are serious limitations on such studies in the circumstances under which we work. Should other investigations have better opportunities for experimental work in monkeys consideration should be given to pursuing further some of these problems

Relationships as revealed by serological tests

As pointed out in Chapters 5 and 8, no differences were noted between strains of treponemes in respect of the titer of Wassermann antibody developed

Likewise, no clear differences between strains were detected by studies made with the treponemal immobilization and the treponemal agglutination tests The technique of the quantitative TPI test is not sufficiently accurate to obtain results which are entirely satisfactory from the standpoint of reproducibility. The TPA test on the other hand utilizes the same antigen in successive tests with satisfactory reproducibility of results Even under these circumstances no constant differences were noted Type antigens for the agglutination test were prepared from syphilis, yaws, bejel and cuniculi strains; sera from rabbits infected with most of the recently isolated strains were tested against these antigens. While the agglutinating titer varied from serum to serum there was no pattern of variation in titer according to the type of antigen employed, with the possible exception of the cuniculi antigen Determination of both immobilization and agglutination titers on sera from cuniculi-infected animals revealed in general higher titers with cuniculi antigens and lower titers with syphilis, yaws and bejel antigens The trend was toward a reverse relationship in respect of the sera from animals infected with strains of syphilis, yaws and bejel and endemic syphilis treponemes

Comparative Susceptibility of Strains of Treponemes to Penicillin

As explained in Chapter 9, both *in vivo* and *in vitro* methods were used to determine the susceptibility of newly isolated treponemal strains to penicillin While these methods are subject to fairly wide variation, the studies in question may be briefly summarized by stating that the experimental data failed to reveal significant differences in susceptibility to penicillin among the strains tested

The experimental methods were regarded as accurate within a threefold limit of variation. Since most treatment schemes with penicillin make use of an excess of the drug the tentative conclusion is drawn from these experimental results that a treatment scheme which has been demonstrated to be therapeutically effective in man in one disease syndrome will be equally effective in other treponemal syndromes.

Data on cross-protection among yaws strains are much less extensive, but what data there are suggest that there is good reciprocal immunity among yaws strains.

Most of our recently isolated strains have been tested against the Nichols strain Data for any one strain are perhaps inadequate to permit definitive characterization of the immunological relationship of that particular strain. But when strains from a given clinical syndrome or a given area are considered as a group (Table LVII, page 223) the results of these tests become more significant.

For example, the Nichols strain without exception showed a high degree of cross-protection against all the syphilis strains. Rabbits infected with the Nichols strain, however, exhibited a much lower degree of immunity when challenged with a group of 6 newly isolated yaws strains, although against 2 of these strains there was good protection in the limited number of animals tested It is interesting too that protection was less than complete against each of the 3 bejel strains employed for challenge, while against each of 2 endemic syphilis strains, and each of 2 dichuchwa strains there was a high order of cross-immunity.

With respect to cuniculi strains, while significant degrees of cross-immunity existed between 2 of these strains tested and the Nichols strain and other syphilis and yaws strains, the cross-protection was not of a high order.

In order to study the antigenic relationship between culture strains of treponemes and pathogenic varieties, rabbits were given a long series of injections of the former organisms and challenged with small doses of the Nichols strain of pathogenic treponemes. No significant degrees of cross-immunity were observed.

While, therefore, no precise patterns emerge from all the cross-immunity tests described in Chapter 8, some outlines are clearly detectable. Just as strains of treponemes differ in their capacity to evoke a particular disease picture in rabbits or hamsters, so they may differ in their capacity to evoke cross-protection in a rabbit host; moreover, as a generalization these variations in antigenic capabilities go hand in hand with those biological characteristics which determine tissue reaction

Before leaving this subject special comment should be made on the arresting fact that a naturally occurring treponematosis of rabbits—if such a term is permissible in connexion with an animal disease—not only is closely related in many biological characteristics to all the treponematoses known among humans, but this cuniculi infection is also capable of evoking significant degrees of cross-immunity to infection with syphilis and other pathogenic treponemes

As noted in Chapters 2 and 8 we have been much interested in the potentialities of this relationship, and have sought to explore certain practical problems incident thereto. The studies in question have by no means been

and host. Less well understood but probably none the less real are the inherent capabilities for adaptation on the part of the treponeme, so that caution must be exercised in assuming that a balance established in favor of the host by artificial means will remain thus for long periods of time; constant vigilance concerning the mutational capabilities of the treponeme should be the order of the day.

That adaptive mechanisms reside in both parasite and host is suggested by a study of the clinical and epidemiological features of the various recognized treponemal syndromes, and support for this basic biological observation is forthcoming from the results of laboratory studies. There can be no question that strains of treponemes isolated from patients with different syndromes do differ in certain fairly stable biological characteristics

The outcome of this interplay between parasite and host over centuries, in situations which differ in their physical and ecological characteristics, has brought about not only modifications in the human host—however slight and difficult to measure—but also changes in the treponeme. Perhaps the most astonishing aspect of this situation is not that there are differences in treponemes, but that during the countless generations of straight-line descendancy strains have survived in which these differences are slight

The pathogenic treponemes which we have been able to study in the laboratory both *in vivo* and *in vitro* are very closely related in their essential biological characteristics, in the disease picture they invoke in man and in experimental animals, in their immunological features, and in their reaction to antibiotics As mentioned above, however, certain relatively stable differences have been observed These differences relate particularly to the kind of lesions invoked in rabbits, the disease picture in hamsters, and certain immunological patterns as determined by challenge inoculation of rabbits

On the basis of those criteria, strains of treponemes from various parts of the world have been placed with some unexplained overlapping into one of the three following categories· (*a*) the S-type, comprising most but not all of the strains isolated from patients with the classical disease syndrome of venereally acquired syphilis, (*b*) the Y-type, comprising most but not all of the strains isolated from patients with the classical disease syndrome of yaws; and (*c*) the M-type, which in the foregoing characteristics occupies an intermediate position between the S and Y types The M-type comprises most but not all of the strains isolated from patients with disease syndromes of bejel, endemic syphilis, and locally designated syndromes of endemic treponematosis, such as dichuchwa Perhaps strains of treponemes from naturally occurring cuniculi infection in rabbits should be placed in a fourth category, which we might designate the C-type Serological tests, including the treponemal agglutination and the treponemal immobilization tests, have failed to reveal serological differences between the S, Y and M types, and have demonstrated only qualitative differences between these three

It should be pointed out that the data on the upper limits of the effective dose of penicillin are perhaps more accurate than similar data bearing on the least amount of the antibiotic that is effective In other words, while all strains responded well to that dosage level which was found to be effective for most strains, it is possible that some strains might respond well to substantially smaller amounts of penicillin.

III. CONCLUSIONS

In this monograph we have been concerned with studies on the fundamental biology of the treponematoses, particularly those which have been carried out at the International Treponematosis Laboratory Center, Johns Hopkins University While these studies cover a wide range, obviously unequal attention has been given to some facets of the over-all problem. Even within this context, however, there emerges a picture, vague in many details, but nevertheless whole as regards the basic biology of this great group of diseases.

This picture differs in certain important respects from that which might have been drawn a decade or two ago. The main effect of this re-orientation is to bring the treponemal diseases more closely in line with other infectious processes. Thus research on the treponematoses has not diverged from the main stream of medical and biological research, but has formed an integral part of it, taking from other fields of investigation and making contributions to them. We may anticipate that this fruitful exchange of new knowledge will continue

It is clear that, except in the case of pinta, a treponemal disease which we have not been able to study for lack of successful reproduction in laboratory animals, treponemes from treponemal syndromes when established in experimental hosts behave according to a common pattern, which varies in detail but is essentially similar in its broad outline A relatively few organisms, perhaps even a single treponeme, can produce infection. Multiplication ordinarily occurs at a regular although a comparatively slow rate, and grossly discernible lesions are produced by the mass of growing treponemes. The immune reaction of the host, which in its essential features seems to be similar to that invoked during the course of other infectious diseases, begins to develop early during the course of infection, increases in a leisurely fashion, and reaches a degree in which it is serviceable to the host, but inadequate to rid the host completely of the invading treponemes. The balance struck between host and parasite may be exquisitely fine, although wide fluctuations in this balance are probably the rule rather than the exception.

Many more or less extraneous factors affect this balance; some of them—temperature, for example—occur as a result of the natural environment, others, such as the antibiotics, may be artificially interposed between parasite

and host. Less well understood but probably none the less real are the inherent capabilities for adaptation on the part of the treponeme, so that caution must be exercised in assuming that a balance established in favor of the host by artificial means will remain thus for long periods of time; constant vigilance concerning the mutational capabilities of the treponeme should be the order of the day

That adaptive mechanisms reside in both parasite and host is suggested by a study of the clinical and epidemiological features of the various recognized treponemal syndromes, and support for this basic biological observation is forthcoming from the results of laboratory studies. There can be no question that strains of treponemes isolated from patients with different syndromes do differ in certain fairly stable biological characteristics.

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types and the C-type. It is apparent that the tests detect antigens which are common to the several types.

On the basis of limited evidence we believe that a fundamental biological difference between these types does exist, residing in the character and the amount of capsular mucopolysaccharide that each strain produces. Under laboratory conditions of animal passages which favor the treponeme over the host, we have noted an increase in production of mucoid material and a shift toward the S-type reaction To what extent such shifts either toward the S-type on the one hand or toward the Y or C types on the other may be taking place in nature can be only a question for speculation.

What of the future? What main lines of basic inquiry does it seem profitable to pursue? The field of prophesy is traditionally unrewarding for the scientist, and yet perhaps one may say with some degree of confidence that without a minimum of laboratory research in this broad field we can expect little accretion to our knowledge of the biology of the treponematoses. As a corollary, it may be predicted on the basis of experience that continuing investigation at the fundamental level by alert and imaginative investigators carries always the germ of unanticipated and possibly highly significant advances in knowledge, advances which may contribute to biology and medicine as a whole, as well as to the conquest of the treponematoses.

Within this context, we suggest that four main lines of investigation may be worthy of continued exploration

1. Study of the adaptive and mutational patterns of the pathogenic treponemes. Particular attention might be paid to the potential impacts of antibiotics and radioactivity.

2. Immunochemical studies of pathogenic treponemes, with particular reference to identification of serologically active antigenic components both in the body of the treponeme and in the capsular material, with a view to improved methods for demonstrating group- or species-specific antibody.

3 Study of the nature of Wassermann antibody and the mechanism of its production, with a view to elucidating the significance of its occurrence in treponemal and other chronic disease processes.

4. Continued efforts to cultivate pathogenic treponemes *in vitro*.

APPENDICES

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APPENDIX 1

THE SOURCES AND ISOLATION OF STRAINS

In the isolation of treponemal strains from different parts of the world the International Treponematosis Laboratory Center has had most willing and helpful collaboration from physicians and scientists in many countries The following notes which refer to numbers listed in Table Ip can give only feeble credit to those who assisted in this undertaking, doubtless the names of some individuals who contributed time and effort have been unintentionally omitted Without the assistance of the Venereal Diseases and Treponematoses Section of WHO, under the direction of Dr T Guthe, in the coordination of research, many of these investigations would not have been possible

Details concerning unsuccessful isolations are not included.

(1) *Syria A strain*—Transfers made by Dr John C Hume and Dr Emil Rizk from lip lesion of patient A M, aged 6 years, who had typical bejel lesions of about 3-4 months' duration The animals were inoculated on 6 May 1950, and arrived in this laboratory on 17 May 1950

(2) *Syria B strain*—Transfers made by Dr Hume and Dr Rizk from vulva lesion and lower lip lesion of patient W D, a child of unstated age Lesions were regarded as typical bejel The animals were inoculated on 6 May 1950, and arrived in this laboratory on 17 May 1950

(3) *Bosnia A strain*—Transfers made by Dr E I Grin, who wrote as follows "I selected three typical cases with early secondary syphilis lesions All three are peasants, residents of the north-east part of Bosnia where syphilis is endemic" All patients were from remote villages This strain was isolated from a 35-year-old male patient K A S (No 86/50), who had mucous patches under the tongue, on the tonsils and papular secondary lesions on the face, less on the trunk and extremities, some of which, however, were pustular, and moist condyloma on the genitalia Serological tests—Kahn and MKR II—were positive Material for inoculation was taken from an ulcer on the shaft of the penis Darkfield showed many treponemes Inoculations were made on 5 September 1950, and the animals arrived in this laboratory on 22 September 1950

(4) *Bosnia B strain*—Transfers made by Dr E I Grin The comments made on Bosnia A strain apply here, too This strain was isolated from patient N G G, a 38-year-old male, whose wife and three children also had early syphilis Patient had secondary syphilis lesions in mouth and on skin of scrotum Darkfield-positive material was collected from scrotal lesions Kahn and MKR II tests were strongly positive The animals were inoculated on 5 September 1950, and arrived in this laboratory on 22 September 1950

(5) *Baghdad A strain*—Transfers made by Dr M Tuomioja and Dr E H Hudson from patient S H, a 20-year-old auctioneer, single, living in Baghdad There was a history of sexual exposure about two months previously and physical examination showed a primary syphilitic lesion on the penis with typical regional adenitis Material from the lesion was darkfield positive and blood serological tests (Laughlen, Wassermann, Kahn and Rein-Bossak) were strongly positive The animals were inoculated on 30 December 1950, and received in this laboratory on 6 January 1951

(15) *Samoa E strain*—Same note as for (14), except transfers were made from patient M , a 3-year-old male with typical generalized yaws Material was obtained from frambesiform lesions

(16) *Samoa F strain*—Same note as for (14), except transfers were made from patient M , a 4-year-old male with typical generalized yaws lesions.

(17) *Bechuanaland C strain*—Transfers made by Dr J F Murray from patient G G (No R N 8465), a Bantu female, aged 22 years, resident in the Suping district of Bechuanaland This patient had vulva and anal condylomata and mucous patches on the soft palate and fauces of about 4 months' duration These lesions were darkfield positive and were regarded as typical of the non-venereal treponematosis syndrome designated " dichuchwa ", although since the patient was an adult, the presence of venereally acquired disease could not be ruled out Inoculations were made on 3 April 1954, and the animals were received in this laboratory on 8 April 1954

(18) *Bechuanaland D strain*—Transferred by Dr J F Murray from patient M M (R N 8458), a Bantu female 6 years of age residing in the Molepolole district of Bechuanaland This patient showed mucous patches on fauces and buccal mucosa of about 2 months' duration Darkfield examination of the lesions was positive and the case was regarded as typical of non-venereal treponematosis or " dichuchwa " The animals were inoculated on 3 April 1954, and arrived in this laboratory on 8 April 1954

(19) *Gambia A strain*—Transfers made by Dr A H Davies from B K (Case No 7) a 4-year-old female of the Losola (Fula) tribe in Gambia, West Africa The patient had an ulcer on the buccal mucosa and inner surface of the lip which had been present one year The lesion was darkfield positive and was regarded as typical of the non-venereal treponematosis syndrome known as " siti " Transfers were made on 9 July 1955 to two hamsters, and the animals were received in this laboratory on 23 July 1955

(20) *Gambia B strain*—Transfers made by Dr A H Davies from patient S S (Case No 8) an 11-year-old member of the Tukalor (Fula) Tribe, residing in Diganteh Central Division, Gambia The patient had had a small rounded ulcer on the buccal surface of the upper lip for about one year The lesion was darkfield positive and was regarded as typical of the non-venereal treponematosis syndrome known as " siti " Transfers were made on 9 July 1955, and the animals were received in this laboratory on 23 July 1955

(21) *Gambia C strain*—Transfers were made by Dr A H Davies from T S , a 4-year-old male of the Tukalor (Fula) Tribe who had had darkfield positive ulcers on the upper lip for 10 months The inoculations were made on 9 July 1955, and the animals were received in this laboratory on 23 July 1955

(22) *Gambia D strain*—Transfers made by Dr A H Davies from O G (Case No 10), a 4-year-old male who had had sore areas on the face, upper lip, right shoulder and scrotum for about one year Transfers were made from the lip lesion, which was darkfield positive This was regarded as a typical case of " siti " Inoculations were made on 9 July 1955, and the animals were received in this laboratory on 23 July 1955

We wish to thank Dr James A McFadzean, who made the initial arrangements for the transfers of the Gambia strains

(6) *Baghdad B strain*—Transfers made by Dr M Tuomioja and Dr E. H Hudson from patient J A, an unmarried male fisherman of about 40 years of age There was a history of repeated sexual exposure and a penile lesion of about 15 days' duration Examination showed a typical primary syphilitic lesion on the glans penis with regional lymphadenopathy Material from the lesion was darkfield positive and blood serological tests (Laughlen, Kahn, Wassermann, and Rein-Bossak) were strongly positive. Transfers were made on 30 December 1950, and the animals were received in this laboratory on 6 January 1951

(7) *Samoa A strain*—Transfers made by Dr M J Marples from patient No. 11—J, an 18-month-old male resident of Apia, West Samoa. The patient had frambesiform lesions scattered in large numbers over the body Material for transfer was taken from a large frambesioma on the right thigh and from two smaller lesions on the left ankle After dilution with saline, the material was darkfield positive, showing about three treponemes per field The animals were inoculated on 12 January 1951, and received in this laboratory on 25 January 1951

(8) *Chicago strain* Transfers made by Dr T B Turner and Dr J Rodriguez from patient W McD (No 3163), a Negro male about 25 years of age There was a large, greatly indurated annular primary syphilitic lesion on the prepuce of about 3 weeks' duration Material collected from the lesion showed approximately 12 000 000 treponemes per ml on darkfield examination Transfer was made on 9 February 1951, and the animals were received in this laboratory on 17 February 1951.

(9) *Indonesia B strain*—Transfer made by Dr Huang-Ying Li from patient S a female of about 11 years of age, who had had frambesiform yaws lesions for approximately one month Residence, Kemajoran Bendungan, Djakarta. A brother and a sister also had early yaws at the time Transfers were made from typical frambesioma on cheek, darkfield on inoculated material showed 8-10 treponemes per field The animals were inoculated on 3 March 1951, and received in this laboratory on 8 March 1951.

(10) *Haiti A strain* Transfer made by Dr S Levitan from patient M. E (No 480), a male aged 9 years, resident in Commune de Bainet. The patient had typical generalized frambesiform yaws, with history of initial lesions 6 months previously Material transferred was darkfield positive The animals were inoculated on 7 March 1951, and received in this laboratory on 10 March 1951

(11) *Haiti B strain*—Transfer made by Dr S Levitan from patient J L. S. (No 482), an 11-year-old male resident of Commune de Côtes de Fer Patient had typical generalized frambesiform yaws with history of initial lesion about 5 weeks previously Lesions on the lower abdomen were used for transfer The animals were inoculated on 7 March 1951, and arrived in this laboratory on 10 March 1951

[illegible] G [illegible] hes [illegible] this laboratory on [illegible] May 1951.

(13) *Mexico A strain*—Transfers made by Dr J. Olarte from patient G A, an 18-year-old male who had a typical primary syphilitic lesion of 10 days' duration The transferred material was darkfield positive. The animals were received in this laboratory on 17 January 1953.

(14) *Samoa D strain*—Transfers made by Dr M J. Marples from patient I, a 7-month-old male resident of Apia, Western Samoa, with typical generalized lesions of yaws Transfers were made from frambesiomas, which were darkfield positive The animals were inoculated on 24 January 1953, and arrived in this laboratory on 30 January 1953

INDEX

APPENDIX 2

PREVENTION AND TREATMENT OF LABORATORY ACCIDENTS

It appears that pathogenic treponemes do not lose their virulence for man on repeated animal passage (see Chapter 7) There is, therefore, an inherent risk involved in experimentation with this group of organisms, although the risk can be minimized by attention to details which at first glance might seem trivial In our laboratory most accidents involving the risk of treponemal infection to laboratory personnel have arisen from the following circumstances (a) inadvertent use of unplugged pipettes; (b) use of syringes to which the needle is not firmly attached and which becomes separated under pressure, and (c) piercing of the operator's hand with an infected needle when an animal suddenly struggles

In regard to the last eventuality, even with ordinary care an animal may struggle or jump suddenly during an injection For intratesticular inoculation rabbits should be held by an assistant, the ears and the scruff of the neck being firmly grasped with one hand, and the hind legs with the other In all inoculations and bleedings, the syringe should be held in such a manner that the needle is never pointed at the hands of the operator or his assistant Difficult inoculations are more safely performed under nembutal anesthesia in the case of rabbits or ether in that of hamsters

The availability of penicillin as a relatively non-toxic treponemicidal agent has materially altered the approach to the clinical management of laboratory accidents with these organisms It now seems wise to give treatment in cases which would not have warranted the risk of arsenical therapy Nevertheless, the use of penicillin is not without some attendant risk, and there remains the need for care in handling treponemes in order to avoid accidents

Laboratory accidents with treponemes should be dealt with by the general principle that if any parenteral treatment is warranted, sufficient should be given to destroy all the treponemes which may have entered the body The precise manner of treatment should [illegible] on the one hand, and [illegible]

[illegible] ing the laws of chance and weighing the risks of infection against the risks of treatment complications

[illegible] of a treponeme [illegible] th the unbroken skin involve relatively [illegible]

[illegible] possibility that treponemes have entered the body, in order to arrive at a decision on the advisability of treatment

INDEX